DETERMINATION OF SOME FUNCTIONAL PROPERTIES AND BIOPROTECTIVE POTENTIAL OF METABOLITES OF NATIVE LACTIC ACID BACTERIA ISOLATES

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ABSTRACT

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In the relevant study, postbiotics were obtained from 10 native Lactic Acid Bacteria (LAB) and were analyzed for functional and antimicrobial properties. These species were from Lactobacillus (*Lb. plantarum*, *Lb. paraplantarum*, *Lb. coryniformis*, *Lb. brevis*, *Lb. helveticus*, and *Lb. bulgaricus*, Enterococcus (*E. faecium* and *E. faecalis*), Lactococcus (*Lc. lactis*), and Streptococcus (*St. thermophilus*) genera. The growth curve of all LAB was determined by measuring their optical densities. To obtain postbiotics, Lactobacillus species were anaerobically grown in MRS broth (37 ºC), and other LAB (enterococci, lactococci and streptococci) were grown aerobically (30 ºC) in M17 broth. Later, the bacterial pellet was separated by centrifugation, and postbiotics were taken to analyze for their functional properties such as antioxidant activity, antimicrobial activity, phenolic content, and organic acid content. These postbiotics were added to pasteurized milk (at 78 °C for 2 min) with and without *E. coli* (ATCC 25922) to check their bio-preservative effect during the shelf-life (1,5, and 10^{th} days) of pasteurized milk. According to the obtained results, high antioxidant activity was shown by postbiotics of *Lc. lactis*, followed by that from *E. faecalis* while phenolic content was highest in postbiotics of *Lb. plantarum* and *E. faecalis*. In pasteurized samples of milk, although all postbiotics caused a decrease in *E. coli* (ATCC 25922) count, this decrease was significant in the presence of postbiotics from *Lc. lactis*, *E. faecium*, and *Lb. helveticus* which proved them to be having highest bio-preservatory activity. The TAMB count was reduced significantly by postbiotics of *Lb. helveticus* but was increased in samples containing postbiotics from *Lb. plantarum* and *E. faecalis*. While bio-preservative activity was shown by all postbiotics, considering the effect on pH, TA %, and rheology of pasteurized milk it was concluded that not all postbiotics were suitable for addition in pasteurized but could be evaluated for other dairy products as a bio-preservative.

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Key Words: Lactic Acid Bacteria, Postbiotics, Metabolite, Cell Free Supernatant, Bio-preservation, Pasteurized milk

ÖZET

Yüksek Lisans Tezi

YERLİ İZOLAT LAKTİK ASİT BAKTERİLERİ METABOLİK BİLEŞENLERİNİN BAZI FONKSİYONEL ÖZELLİKLERİ İLE BİYOKORUYUCULUK POTANSİYELİNİN BELİRLENMESİ

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Postbiyotikler, bakterilerin büyümeleri sırasında ürettikleri metabolitlerdir. Bunlar hücresiz süpernatanlar (CFU) olarak elde edilir ve faydalı proteinleri, peptitleri, organik asitleri, SCFA'ları, bakteriyosinleri ve/veya diğer hayati bileşenleri içerir. Bu postbiyotik metabolitlerin, gıda ürünlerine eklendiğinde temel anti-biyofilm, antimikrobiyal, antioksidan, anti-diyabetik, anti-inflamatuar ve biyokoruyucu aktiviteleri gerçekleştirdiği kabul edilir. Postbiyotikler genellikle sağlıklı ve faydalı laktik asit bakterilerinden elde edilir. Canlı bakterilerin doğrudan kullanımıyla bağlantılı birçok zorluk vardır. Gıda endüstrisi açısından, kontrolsüz fermantasyonda doğrudan bakterilerin kullanılması asitlikte artışa neden olabilir ve ayrıca UHT gibi yüksek sıcaklıkta işlem yararlı bakterileri öldürebileceğinden ısıl işlemi değiştirebilir.

Bu tez çalışmasında 10 adet yerli Laktik Asit Bakterisinden (LAB) postbiyotikler elde edilerek fonksiyonel ve antimikrobiyal özellikleri analiz edilmiştir. Bu türler Lactobacillus (*Lb. plantarum, Lb. paraplantarum, Lb. coryniformis, Lb. brevis, Lb. helveticus* ve *Lb. bulgaricus*), Enterococcus (*E. faecium* ve *E. faecalis*), Lactococcus (*Lc. lactis*) ve Streptococcus (*St. thermophilus*) cinslerindendir.

Amaç, maksimum miktarda bakteri metaboliti elde etmekti ve bakteriler durağan fazda yüksek sayıda metabolit üretilmektedir. Her bir bakterinin durağan fazı, log döngüleri gözlemlenerek belirlendi. Her bakteri için log döngüsü, her 2 saatte bir optik yoğunluklar (OD) kontrol edilerek belirlendi. Durağan faz tüm LAB türleri için not edildi. Postbiyotik elde etmek için, Lactobacillus türleri 37 ºC'de MRS besiyerinde anaerobik olarak geliştirildi. Streptococcus, Lactococcus ve Enterococcus LAB türleri, durağan fazlarına ulaşana kadar 30 ºC'de M17 besiyerinde aerobik olarak geliştirildi. Postbiyotikleri ayırmak için bakteri kültürleri, peleti hücresiz süpernatan veya postbiyotiklerden ayırmak için 15 dakika boyunca 8000 rpm'de santrifüj edildi.

Postbiyotikler fonksiyonel özellikleri açısından ayrı ayrı analiz edildi. Fenolik içerikler, Folin-ciocalteu reaktifi ile Na_2CO_3 (%20) kullanılarak analiz edildi ve gallik asit (mg GAE/mL) ile karşılaştırıldı. Tüm postbiyotiklerin antioksidan aktiviteleri DPPH testi kullanılarak gözlendi ve troloks standardı (TEAC mM/mL) ile karşılaştırıldı. Altı patojen bakteriye (*Staphylococcus aureus* (ATCC 25923), *Mycobacterium tuberculosis* (ATCC 7644), *Escherichia coli* (ATCC 25922), Bacillus cereus (ATCC 14579*)*, *Salmonella enterocolitica* (ATCC 14028), *Enterococcus faecalis* (ATCC 29212*)*) karşı agar kuyusu difüzyon yöntemi ile antimikrobiyal aktiviteler kontrol edildi. Her bir postbiyotiğin organik asit profili HPLC ile ve 0.013N H_2SO_4 mobil faz kullanılarak belirlendi.

Biyo-koruyucu rolünün belirlenmesi için postbiyotik 100 mL pastörize sütte (78 °C'de 2 dk) biyo-koruyucu etkilerinin belirlenmesi amacıyla *E. coli* içeren ve içermeyen pastörize süte eklenerek raf ömrü boyunca (1, 5 ve 10 günler) mikrobiyolojik analizlere tabi tutuldu. Karşılaştırma için iki kontrol numunesi hazırlandı (biri sadece pastörize süt ve diğeri patojen *E. coli* içeren). Fizikokimyasal analizler depolamanın 1,5 ve 10 günlerinde yapıldı ve pH, TA %, TS % ve reolojik faktörler analiz edildi. Biyokoruyucu rol, patojen *E. coli*'ye karşı kontrol edildi. 100 mL pastörize süte 200 µL postbiyotik ve 100 µL *E. coli* kültürü ilave edildi ve 4ºC'de bekletildi. Depolamanın 1, 5 ve 10. günlerinde TAMB ve *E. coli* sayımı için örnekler gözlendi.

Elde edilen sonuçlara göre, *Lb. paraplantarum*'dan elde edilen postbiyotikler en fazla fenolik içerik sayısına (0.72 ± 0.03) sahipken, bunu *E. faecalis*'in postbiyotikleri (0.66 ± 0.04) izlemiştir. *Lb. plantarum* (3.67 ± 0.25) postbiyotikler için yüksek antioksidan aktiviteler gözlendi, bunu *E. faecalis* (3.60 ± 0.11) ve *Lc. lactis* (3.58 ± 0.18) postbiyotikleri izledi. Yüksek miktarda fenolik içerik, *E. faecalis*'in postbiyotiklerinde yüksek antioksidan aktiviteleri ile bağlantılı olabilir. Agar-well difüzyon yöntemi ile belirlenen antimikrobiyal aktivite, *E. coli* için önemli inhibisyon zonları gösterdi. Lactobacillus türlerinden postbiyotiklerle tedavi edildiğinde antimikrobiyal aktivite önemli ölçüde gözlenmiştir. Enterococcus, Lactococcus ve Streptococcus türlerinden postbiyotikler, *M. tuberculosis*'e karşı iyi antimikrobiyal aktiviteler gösterirken, Lactobacillus türlerinden postbiyotikler bu patojene karşı herhangi bir antimikrobiyal aktivite göstermedi. Tüm postbiyotikler *E. coli*'ye karşı antimikrobiyal aktivite gösterdiğinden, antimikrobiyal aktiviteyi gözlemlemek için denenmiş patojen olarak alınmıştır. Tüm postbiyotiklerde farklı miktarlarda farklı organik asitler mevcuttu. Sitrik asit diğer asitlere göre daha düşük miktarda tespit edilmiştir. Pirüvik asit, *Lb. brevis*'in postbiyotiklerinde yüksekti, Enterococcus türlerinin postbiyotiklerinde çok az miktarda mevcuttu. Lactobacillus bakterilerinden elde edilen postbiyotikler daha düşük süksinik asit üretti. Laktik asit ve asetik asit, tüm postbiyotiklerde daha büyük miktarlarda mevcuttu. Formik asit, Enterococcus bakterileri tarafından yüksek miktarda üretildi, ancak Lactobacillus bakterileri daha düşük miktarlarda üretildi. Propiyonik asit ve bütirik asit, Lactobacillus bakterileri tarafından az miktarda üretildi, ancak Enterococcus ve

Streptococcus bakterileri herhangi bir bütirik asit üretemedi. Ayrıca tüm postbiyotiklerde asetoin ve diasetil de tespit edildi.

Postbiyotiklerin potansiyel biyokoruyucu rolünü gözlemlemek için, deneysel bir süt ürünü olarak pastörize süt alındı. Postbiyotik içeren tüm örneklerde *E. coli* sayısında kademeli bir azalma gözlendi, ancak *E. faecium* ve *Lb. helveticus*'tan elde edilen postbiyotikler tarafından önemli bir azalma gösterildi. Toplam Aerobik Mezofilik Bakteri sayımı (TAMB) açısından, *Lb. helveticus*'tan elde edilen postbiyotikler TAMB için minimum log sayısı gösterirken, *Lb. plantarum* depolamanın 10. gününden sonra TAMB sayısında artış gösterdi. LAB türlerinden elde edilen tüm postbiyotikler *E. coli* sayısında azalma gösterdiğinden, bu bakteri kültürleri tarafından üretilen postbiyotiklerin pastörize süt örneklerini kontamine edebilecek *E. coli* kolonilerine karşı etkili bir şekilde yardımcı olduğu kabul edilmektedir. Bazı postbiyotikler ayrıca aerobik mezofilik bakteri sayımını kontrol etmede yardımcı oldu. Postbiyotik içeren tüm örneklerin fizikokimyasal özellikleri ve biyokoruyucu aktiviteleri karşılaştırıldığında, tüm postbiyotiklerin pastörize sütün biyokoruntunda kullanılamadığı, bunun yerine fonksiyonel özellikleri nedeniyle diğer süt ürünlerinde kullanılabileceği görülmüştür. pH, TA % ve reolojik parametrelerdeki değişiklikler karşılaştırıldığında, *Lc. lactis* ve *Lb. helveticus*'tan elde edilen postbiyotiklerin pastörize süt için en uygun postbiyotikler olduğu belirlendi. Postbiyotikler çok sayıda metabolitten oluştuğundan, pastörize sütte yüksek antimikrobiyal aktivite ve biyokoruyucu rolden sorumlu olan postbiyotik metabolit tiplerini belirlemek için kısa bir çalışma yapılması tavsiye edilir.

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Anahtar Kelimeler: Laktik Asit Bakterileri, Metabolit, Postbiyotikler, Hücre bağımsız süpernatant, Biyokoruyucu, Pastörize süt

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1. INTRODUCTION

Humans and microorganisms have been indigenous to this earth for thousands of years. Microbes are an essential part of the ecosystem. They perform various functions like dissolving the decaying matter, giving texture and taste to food commodities via fermentation process, and keeping the environment clean, etc. The human gut comprises thousands of microbial colonies. These microorganisms carry out a variety of functions in the gut, especially the small intestine. Microorganisms comprise bacteria, fungi, viruses etc. Many virus species are regarded as contagious and harmful for human beings, causing many diseases like hepatitis, influenza, measles and covid to name a few. Only a small fraction of viruses called bacteriophages could be utilized for beneficial purpose for humans. Fungi which include yeasts and mold species could be both beneficial and harmful. For example; mushrooms, used in cuisines is a fungus while rusts and smuts (that cause rusty color and blackening respectively of maize and other plants) are also a fungus.

In the same way, bacteria (depending upon type and specie) could be regarded as both useful and harmful. Harmful bacterial species include *E. coli* (causing *E. coli* infection), *Listeria monocytogenes* (listeriosis and bio-film formation in foods), *Mycobacterium tuberculosis* (causing tuberculosis) etc. is responsible for deteriorating the food commodities that results in food wastage. Useful bacteria including a variety of LAB, Leuconostoc bacteria, and Bifidobacterium species, plays a vital role in the fermentation of dairy products (yogurt, kefir, and cheese etc.). They help not only in the production of innovative food products but also aids in enhancing their shelf-life (Castellone et al., 2021).

Thousands of bacteria resides throughout the human GI tract. These bacteria are divided into probiotics, also known as “good bacteria” and prebiotics which is a food for probiotics. Probiotics are gut friendly bacteria which perform various functions in the human body. These functions include aiding in digestion, boosting immunity, production of various vitamins etc. (Cicenia et al., 2014). While the probiotics are always regarded as safe for consumption and come under the category of GRAS (Generally Recognized as Safe). Yet these are a few concerns arising with the use of probiotics in unidentified concentration. For immunocompromised population, including children (Land et al., 2005), pregnant women, and elderly (Abdelazez et al., 2022; Jain, McNaught, Anderson, MacFie, & Mitchell, 2004), the unprescribed use of probiotics could cause destructive changes in natural microbiota in gut and small intestine.

LAB are known for their beneficial properties in many fermented food products. LAB has been known to carry out the breakage of milk sugar (lactose) to lactic acid along with various other components like aromatic compounds, alcohols, acids, SCFAs etc. (Teame et al., 2020). The process is commonly known as fermentation and is crucial for the development of texture and aroma of the food product. In dairy products, LAB is essentially used to carry out fermentation. These products include yogurt, kefir, cheese, etc. Some beneficial bacteria like LAB are widely accepted as beneficial, so a little research is carried out in terms of their probable harmful effects (Marteau, 2001). Some studies showed that the cause of disease prolongation comes from unchecked consumption of probiotics and their further colonization in gut. According to Tommasi et al. (2008), the effect of LAB specie i.e., *Lactobacillus casei* is observed in causing bacteremia in immunocompromised old-aged patient. The blood testing conducted after persistent inability of patient to heal, showed the presence of *Lactobacillus casei* in the blood which was also confirmed with PCR sequencing. Although Lactobacilli is considered to aid in defense against gut pathogens, some studies also showed them as a causing agent in dental carries and also a causing agent of bacteremia in patients already dealing with other ailments. Other probable consequences of unchecked consumption of live bacteria lies with the HGT or Horizontal Gene Transfer that might interfere with the genetic behavior of the host individual (Lerner, Shoenfeld, & Matthias, 2019). The antibiotic resistance genes detected by Rosander, Connolly and Roos (2008) from plasmids of *Lactobacillus reuteri* also confirm the above study. Some studies also link the overgrowth of bacteria in gastrointestinal tract, bloating in stomach and brain fogginess in certain patients.

These above-described concerning effects are more profound in immunocompromised communities like infants, children, pregnant women, older people, and patients. In such cases, it is better to avoid consuming such products because instead of providing positive effects, these bacteria might infer harms to the body. Since these bacteria are mostly present in fermented dairy products it's better to find alternatives that could provide the same benefits but without ingesting the live bacteria.

To get the benefits of LAB and other beneficial bacteria, postbiotics might be present as an effective alternative. The term "Postbiotic" refers to the metabolites that the bacterial species produce during their growth cycle, or the compounds/components introduced in the environment as a result of the bacterial cell lysis. The word "Postbiotic" comes from two words i.e., the prefix - "Post" which means After and "Biotic" which means living or related to living organisms. Hence, the term is described as "Afterlife" of the living organisms. The concept of postbiotics came into being in last few years hence, no official definition of postbiotics. The only definition that could be extracted from the literature is the one given by Salminen et al. (2021). According to ISAPP; postbiotics are lifeless microorganisms with/without their metabolites that present a positive health effect on the host. This differentiates the postbiotic from probiotic which generally constitutes the live microorganisms. Postbiotics sometimes are also wrongly confused with parabiotic which include viable bacterial cells. Whereas postbiotics only refer to as the metabolites produced by the bacteria and/or the dead bacterial cells along with their cellular components.

Since postbiotics are in the form of liquid supernatant or the lyophilized products that contains metabolites so sometimes these are also referred to as the Cell Free Supernatant (CFS) or the Cell Free Extract (CFE) (Moradi et al., 2020). The beneficial bacteria are primarily present in the fermented foods like kefir, yogurt, cheese, sauerkraut or/and other fermented products in which bacteria plays a central role to carry out the fermentation process so many postbiotics are obtained from such bacteria. Also, owing to the probiotic effects in healthy patients, about 90% of postbiotics are produced from processing Lactic Acid Bacteria LAB probiotic bacteria. Some other bacteria species of Lactobacillus, Bifidobacterium, Streptococcus, and some Bacillus bacteria could also potentially be utilized for the production of postbiotic metabolites. In addition to this, some useful yeasts like Saccharomyces spp. are also used to produce postbiotics.

In terms of the food manufacturing, the emphasis has been derived from chemical adulterants and preservatives towards natural ingredients that confer similar properties. To overcome both concerns i.e., human safety (linked risks with consuming live probiotic and other LAB bacteria) and food safety (risks linked with the use of chemicals and consumer awareness), postbiotics are deemed to play an important role. Postbiotics could be an effective way of preserving food commodities not only for products but also for perishable fruit and vegetables. In circumstances where bio preservation is important for consumer safety, adding postbiotics could help in abolishing the manufacturers' concerns regarding shelf-life enhancement. Studies proved the antimicrobial, antioxidant, and antibiofilm properties in various food commodities. Other functional properties provided by postbiotics include anti-inflammatory properties, anti-carcinogenic properties, anti-diabatic properties, anti-hypertensive properties etc. (Aghebati-Maleki, Hasannezhad, Abbasi, & Khani, 2021).

In the performed studies the functionality of postbiotics obtained from native LAB is studied before adding them to pasteurized milk. The *in-vitro* antimicrobial activity, antioxidant activity, phenolic and organic acid contents are evaluated for this aim. The bio-preservatory role is checked by introducing postbiotics in pasteurized milk and the bioactivity is observed at various intervals during 10-day storage.

Main objectives of the study are;

1) Obtaining cell-free supernatant/extract (postbiotics) of native Lactobacillus, Enterococcus, Lactococcus and Streptococcus species used for this study.
2) Detecting the functional properties of obtained postbiotics such as antioxidant and antimicrobial activities as well as phenolic and organic acid contents.
3) Evaluating the bio-preservative role of postbiotics in pasteurized milk.
4) To search and propose healthy and alternative new methods to enhance the shelf-life of dairy products without adding adulterants and chemical preservatives.

2. LITERATURE REVIEW

2.1 Risks Linked with the Use of LAB and Probiotics

Though probiotics are thought to be a safer option for consumption by normal and healthy human beings but at the same time they might possess a threat in immunocompromised communities e.g., infants, elderly, and patients etc. Our GI tract contains millions of bacteria. This indigenous bacterial flora is essential in the form of its certain characterization and amounts. When live bacteria are consumed, it might alter the natural bacteria flora and this change might pose negative effects (Lerner et al., 2019). In the same way, in infants the GI track in quiet sensitive to such bacteria and might cause serious harmful effects. Additionally, many probiotic bacteria contains antibiotic resistant genes and these could be transmitted to the already present pathogen in the gut and might possess health threat (Gueimonde, Sanchez, de los Reyes-Gavilan, & Margolles, 2013).

A study by Rosander et al. (2008), showed the presence of an antibiotic resistance gene in *Lb. reuteri* (ATCC-55730) a common probiotic bacterium. The phenomena of horizontal gene transfer are not uncommon where the genetic information is transferred to other bacteria residing in the same environment. As our gut consists of a bundle of both healthy and harmful bacteria, transfer of such antibiotic factors might raise a problem especially in diseased patients. As the uncontrolled addition of probiotics is done by many food manufacturers which might cause sensitivity to some patients. Acidosis of formally D-lactic acidosis is a condition described by brain fogginess, fatigue syndrome and other neurocognitive conditions. Probiotic bacteria could largely contribute to intensify such conditions as most probiotic produce high amount of D-lactate (Rao, Yu, Tetangco, & Yan, 2018).

2.2 Processing Challenges Linked with Using LAB and Probiotics

LAB are healthy bacteria and when ingested are deemed to provide certain benefits. Dairy is among the most common source of LAB, especially fermented dairy products since they are enriched with lactic acid bacteria and other beneficial bacterial species. At the same time the addition of LAB and probiotic during manufacturing in non-dairy products causes some threat to the shelf-life of commodity. As LAB are live bacteria and are added in known concentrations in food commodities, so, they continue to multiply in case of uncontrolled and unchecked conditions. Another contributing factor which affects the activity of LAB is heat during the processing of products. For example, in milk - during the UHT treatment of milk any viable microorganism is killed which leads to the inactivation of even probiotics or healthy LAB bacteria (Barros et al., 2020).

2.3 Concept and Definition of Postbiotics

The general concept of postbiotics arises from the metabolic secretions of beneficial bacteria that exist in our environment. These secreted metabolites carry many vital components that are deemed to provide the same benefits as that of their source. Hence, most postbiotics are obtained from GRAS status regarded bacteria. These bacterial species mainly include Lactic Acid Bacteria – that is often utilized for fermentation of dairy, fruit, and vegetable commodities. Since LAB forms a major class of beneficial bacteria, almost 90% of the postbiotics are also obtained from them.

The international scientific association of probiotics and prebiotics generally known as ISAPP states that postbiotics are inmate microbes and the components/metabolites produced by them that might impose a positive health effect in human body (Salminen et al., 2021). The word postbiotic is derived by the combination of 2 words i.e., ‘Post’ which means ‘after’ and ‘biotic’ which refers to ‘life’. Hence, it gives the meaning of “afterlife”. Hence, we could define Postbiotics as the main term which includes a large amount of bacterial components, metabolites, enzymes, cell wall fragments etc.

LAB comprises of various bacterial genera including Lactobacillus spp., Lactococcus spp., Streptococcus spp., etc. These are used as starters in the fermentation of many dairy products like yogurt, curd, cheese, kefir, yogurt etc. During fermentation these bacterial species produce various metabolites and components that carry out vital role for food commodity as well as perform activities when ingested. Its utilization is deemed to play a positive role even in immunocompromised populations like infants were these postbiotics help in the development of healthy gut microbiota (Morniroli, Vizzari, Consales, Mosca, & Giannì, 2021). Hence, it is better to define these bacterial metabolites or Postbiotics as soluble factors that the beneficial bacteria secrete during fermentation or either released in the environment during cell lysis (Moradi, Molaei, & Guimarães, 2021).

2.4 Methods for Obtaining Postbiotics

2.4.1 Treatment with elevated temperature

Heat treatment is one of the easiest ways to kill bacterial cells and obtain their postbiotics. Heat treatment of 121 ºC for 15 min is preferably acquired to kill all viable cells (Chen et al., 2013). Since some resistant cells might remain active after heating to ensure the presence of no viable cell, plate count method is carried out to check any remaining bacterial cells.

Choi et al. (2006) suggests the treatment of Lactobacillus bacterial cells at 95 ºC for 1 hour could kill the cells effectively after which various fractions like soluble polysaccharides, proteins and fatty acids could be obtained by ultrasonic disruption of cell for 20 min at 4 ºC. Bharti, Mehta, Singh, Jain & Ahirwal (2015) showed that the heat killed cells at the concentration of 500 µg/mL of *Lb. plantarum* and *Lb. acidophilus* (heated at 95 ºC for 1 hour) showed significant results by inhibiting the proliferation of MCF-7 cell lines causing the breast cancer after 24 hours.

2.4.2 Tyndallization

Tyndallization is a process very similar to heat treatment but with the addition of incubation in-between heating. This treatment evolved from the concept of sterilization that involves heating at continuous intervals to ensure complete destruction of spores (Kim et al., 2012). In a similar way, in tyndallization the bacterial cells are heated at high temperatures followed by incubation time at lower temperatures and again heating.

Studies showed that this type of heating cause deformation of bacterial cells and cause pores in the membrane (Piqué, Berlanga, & Miñana-Galbis, 2019).

2.4.3 Sonication

The process of sonication is mostly used for the destruction of bacterial cell membrane. Since it is deemed that bacterial membrane also exhibit beneficial metabolites and surface components, this method is effectively used for destroying cell membrane. This causes the essential cell components to leak out along with the fragments of bacterial membrane. As described by Ashraf, Vasiljevic, Smith & Donker (2014), the obtained bacterial cell components are obtained by treating the cell pellet with 100 W output with 2 min interval. The process was carried out till 5 sonication cycles over an ice bath. For this purpose, a probe sonicator was utilized and cell's remaining components were separated via centrifugation.

Vale and Mayer (2021) suggests the method of sonication to obtain bacterial cell lysates. The process is done by applying 20 kHz frequency to already obtained *L. rhamnosus* pellet (bacterial cells) for 25 min. The process is done over an ice bath and at the end of the process the soluble supernatant containing lysed cell components was obtained as the final postbiotic.

2.4.4 Induction of high pressure

According to Liu et al. (2021), a high-pressure bacterial cell disrupter was used to disrupt bacterial cells of *Lb. plantarum*. The initial pressure treatment of 5 kpsi was increased to 35 kpsi and cell waste was later removed via centrifugation. The resultant supernatant was proved to be comprised of high radical scavenging components hence, high antioxidant values were observed via using this technique.

2.4.5 Bacterial lysis with radiation

Radiations such as high intensity ultra sound are proven to enhance the emulsification properties of many dairy products and at the same time these radiations disrupt bacterial cell walls. These sound waves ranging from 20 kHz to 500 kHz frequencies are deemed to be affective against many gram-negative bacteria causing pores in their cells thus disrupting the internal components and exposing them in the environment (Guimarães et al., 2019).

2.5 Quantitative or Qualitative Analysis to Identify Postbiotic Metabolites

a) **GC or Gas chromatography**: generally used for both quantitative and qualitative identification of volatile components – including free fatty acids and organic acids. For example; GC – MS technique to identify various postbiotic alcohols, organic acids, ketones, and esters (Ricci et al., 2018).

b) **Spectrophotometer:** it is sometimes used to identify exopolysaccharides, proteins and hydrogen peroxide which are secreted in the environment.

c) **SDS-PAGE:** this technique is commonly used to identify the protein content in the matrix. The study by Liu et al. (2021)identified the abundance of protein content in supernatant of high pressure treated postbiotics which was observed with many intense protein bands.

d) **NMR spectroscopy:** has been used for identifying antimicrobial and antifungal postbiotics.

e) **FTIR:** it is mostly used to qualitatively identify various bacterial metabolites. Amiri, Rezazadeh-Bari, Alizadeh-Khaledabad, Rezaei-Mokarram, & Sowti-Khiabani (2021) identified major postbiotic metabolites from *Bifidobacterium lactis* BB-12. Various peaks formed during the analysis represented the presence of carbonyl esters, high chain fatty acids, EPS, and amide groups in the postbiotics.

f) **Liquid chromatography:** Among chromatographical techniques HPLC is the most common technique that is used to identify many components that includes organic acids, vitamins, polysaccharides, etc. in the sample. A study by Chan, Lau, Lim, Li, & Liu (2021), revealed that LC system coupled with QTOF-MS was effective to identify the metabolic profile of *L. rhamnosus* and *S. boulardii* in fermented coffee brews. The results precisely identify the presence of many postbiotic metabolites including hydroxy-dodecanoic acid, indole 3- lactate, and 2-isopropylmalate etc.

2.6 Types of Postbiotics

Postbiotics can be divided either on the basis of their structure or their functional performance in human and/or food commodities.

2.6.1 Functionally defined postbiotics

2.6.1.1 Postbiotics as antimicrobial agents

When discussing the antimicrobial properties, bacteriocins are regarded as the most effective metabolites to provide antimicrobial activity against many lethal pathogens. Antimicrobial bacteriocins obtained from cell free supernatants of LAB extracted from traditional fermented milk call “Raib” showed high temperature resistance. The antimicrobial activity was unaffected at elevated temperatures of 65 ºC and 95 ºC (Abdelbasset & Djamila, 2008).

Incorporating postbiotics in the packaging material for the purpose of obtaining both anti-microbial and bio-preservative characteristics is an innovative technique in food packaging technology. Yordshahi, Moradi, Tajik, & Molaei (2020) showed the antimicrobial role of postbiotics of lactic acid bacteria. According to the above study, the postbiotics were obtained from *Lb. plantarum* in the lyophilized form. The packaging incorporated these lyophilized postbiotics showed significant reduction in *Listeria monocytogenes* in packaged ground meat. Additionally, this nanocellulose bio-packaging was produced 3-dimentionally by bacteria i.e., *Acetobacter hansenii* and *Komagataeibacter xylinus*. The antimicrobial activity of postbiotics observed via agar well diffusion method showed the inhibition zone ranging from 6.9 to 34.4 %. The suppression of *L. monocytogenes* was dependent upon the concentration of postbiotic used. During the shelf-life analysis, it was observed that postbiotics are heat and pH stable. They diffuse into the inside environment and perform their activity by reducing the pathogen population hence, increasing storage-life.

2.6.1.2 Postbiotics as anti-inflammatory agents

Many species of LAB are reported to have various anti and pro inflammatory characteristics. As described by Ashraf et al., (2014), the metabolites and cell surface components extracted from a number of LAB including strains of *Lb. acidophilus, Lb. rhamnosus, L. lactis, S. thermophilus, Lb. reuteri* etc. possesses various cytokines of both anti and proinflammatory nature. The abundant proinflammatory cytokine produced was TNF-α. In addition to this, all components obtained in the form of surface proteins were recognized for producing high amount of IL-2.

Anti-inflammatory activity of probiotic LAB including *L. casei, L. reuteri*, *L. lactis*, *L. acidophilus* etc. was shown by De Marco et al. (2018), who suggested that supernatants of these probiotics help in production of IL-8 cytokines. *L. lactis* was considered to be more effective in performing anti-inflammatory activity in human HT-29 cells while IL-6 cytokine production was enhanced by the postbiotics of *L. casei*, *L. reuteri* and *L. lactis*. Moreover, these postbiotics also showed significant radical scavenging or antioxidant activities.

2.6.1.3 Postbiotics as anti-diabetic agents

Diabetes is a serious health condition which affects most of the population of the world. It is often caused by insulin resistance which in severe cases could result in organ damage and other cardiovascular diseases. Since this situation is also closely related to lifestyle and diet changes, incorporating components in the diets that have antidiabetic effect is deemed useful.

Postbiotic components, especially GABA like compounds, are often studied for their anti-diabetic properties. Some Lactic acid bacteria of GRAS status like *Lb. brevis* is also deemed to be the higher producer of GABA in the presence of GAD enzyme. According to Abdelazez et al. (2022), some strains of *Lb. brevis* produce adequate quantities of GABA that when incorporated in the diet, could result in the reduction of blood glucose, as well as affect the hypoglycemic activity. These results are effective in the future utilization of postbiotics from *Lb. brevis* for the purpose of inhibiting diabetic complications.

2.6.1.4 Postbiotics as anti-cancerous agents

Postbiotics are enriched in beneficial metabolites which help in preventing various diseases or can reduce the intensity of disease. Heat-killed cells of *Lb. acidophilus* (606) and *Lb. casei* (ATCC-393) were observed to have high anti-cancer activity in in-vitro models. It is deemed that polysaccharides from *Lb. acidophilus* 606 causes apoptosis via fragmenting the DNA in cancer cells (HT-29 cells). The presence of high radical scavenging potential also opens its utilization in food commodities (Choi et al., 2006).

Cell Free Supernatants of CFS taken from *Lb. reuteri* was observed for its activity against progression of tumors and colon cancer stem cells (HT29-ShE). Cell free fractions of *Lb. reuteri* are effective in causing apoptosis of these stem cells as well as intense antiproliferative activity was shown when compared to the control (Maghsood et al., 2020). In some studies, cell free extracts of *Lb. acidophilus* (KP-942831) were observed for their activity against the glioblastoma and breast cancer cell (MCF-7) line. It was observed that CFE of *Lb. acidophilus* had dose dependent inhibitory effect on the MCF-7 cell lines when used in the concentration of 1000 µg/mL (Bharti et al., 2015). The results showed 98% cytotoxic inhibition after 24 hours of incubation. Some studies observed that the reason for anticancer activity is the invasion of metabolites into the cancer cells.

2.6.1.5 Postbiotics as antioxidants

Ability to prevent oxidation of free radicals or antioxidant properties of various LAB are found in many studies. In terms of bio-preservation this property is especially critical as it prevents the environmental factors from affecting the storage life of food commodities. Many species of lactic acid bacteria produced exopolysaccharides that also exhibit high antioxidant properties including bacteria from Lactobacillus and Leuconostoc genus. The DPPH assay showed radical scavenging activity ranging from 2.99 to 3.30 % of polysaccharide extracted from the cell free extracts of various Lactobacillus bacteria (Păcularu-Burada & Bahrim, 2021).

2.6.1.6 Postbiotics as immunoregulatory agents

The bacterial cell surface components (peptidoglycan and lipoteichoic acid) are considered to trigger a sort of immune response when comes in contact with the body. As a result, these components produce anti-inflammatory as well as proinflammatory response in the body. Postbiotics obtained from multiple LAB species show significant reduction of Salmonella infection in targeted host (Chen et al., 2013). According to Vale and Mayer (2021), postbiotics obtained from *L. rhamnosus* are effective in causing immune response against the cells effected with *P. gingivalis*. The same study showed different types of postbiotics i.e., in the form of postbiotics containing the bacterial metabolites – known as spent media, and postbiotics containing bacterial cell wall components that are soluble – also known as cell lysate. Above mentioned both types of postbiotics carry out distinctively different pathway to perform inhibition of *P. gingivalis* pathogen. The above-mentioned study observed that *L. rhamnosus* (Lr-32) spent-medium causes decreased cell response to pathogen while the postbiotics in the form of cell lysate causes increased cell coding and transcription hence, resulting in better pathogen recognition and its removal from the host. Labaneh is a traditional fermented product that resembles yogurt and have high acidity but lower moisture contents. It contains many probiotic and LAB species that might perform different activities. Lactic acid bacteria from labaneh possesses immunomodulatory properties and the heat killed cells of *Lb. rhamnosus* and *S. thermophilus* showed the ability to produce cytokines both Th-1 and Th-2 (Tarique et al., 2022). Postbiotics from *B. breve* and *St. thermophilus* (065) increase in immunomodulatory response via promoting abundance of Th-1 and Th-17 cytokines (Ayechu-Muruzabal et al., 2021).

2.6.1.7 Postbiotics as anti-biofilm agents

Various lactobacillus species produce postbiotics or metabolites that have intensive antimicrobial effect against pathogens and their ability to produce biofilm on the food surfaces. Postbiotics of *Lb. curvatus* and *Lb. plantarum* (strain M.2) contains certain organic acids and bacteriocins that become hurdle against biofilm producing pathogen especially *Listeria monocytogenes*. This antimicrobial activity was intense within the pH range of 1-6. The activity was hugely decreased at neutral pH range. The study showed the full inhibition of pathogen when 80 mg/mL for postbiotics from *Lb. curvatus* and 70 mg/mL for postbiotics from *Lb. plantarum* were used. This information is useful in the future as postbiotics from such bacteria could be utilized in acidic foods more effectively instead of other commodities. At the same time, it was observed that the obtained postbiotics remained intact under intensive heat treatment (M. I. Hossain et al., 2021).

A similar antibiofilm activity was observed by the postbiotic bacteriocins that were obtained from *Lb. paracasei* LS – 6 (obtained from a traditional Chinese yogurt). When planktonic cells of *S. aureus* were treated with these bacteriocins, intense degradation in the cell membrane and destruction of metabolic activity of *S. aureus* was observed. Additionally, antibiofilm property was also disrupted by those bacteriocins (Jiang et al., 2022). It was deemed that utilization of these postbiotics in dairy products could be useful in exhibiting antibacterial properties, hence, could help in enhancing shelf-life of commodities. Postbiotics obtained from *Lactobacillus plantarum* are considered to have significant anti-biofilm properties. The LAB separated from various sources including dairy yogurt and cheese, presents antimicrobial properties against *E. coli*. The *E. coli* U12 were identified for their bio-film formation characteristics. The analysis performed crystal violet and Congo red agar method indicates that precoating with the supernatant of *Lb. plantarum* shows significant anti-biofilm properties (Mekky, Hassanein, Reda, & Elsayed, 2022).

2.6.1.8 Postbiotics as biopreservative agents in dairy and other products

LAB especially obtained from fermented products are known for their shelf-extension properties. These bacteria not only provide the texture, aroma, taste and flavor to the fermented food products but also secrete antimicrobial metabolites that further protect the food from spoilage. The phenomenon is thus carried out through the inhibition of spoilage causing bacteria and/or deactivation of other harmful pathogens by these metabolites. Many individual postbiotic components are also used to inhibit growth of pathogen bacteria in food. One such example is nisin which is added to many dairy products including cheese to enhance its shelf-life and quality (Ibarra-Sánchez, El-Haddad, Mahmoud, Miller, & Karam, 2020).

LAB is known for producing various metabolites i.e., postbiotics (these includes bacteriocins, organic acids, etc.) that compete with harmful bacteria and restrict their activity. In aquatic food, the antimicrobial activity of various metabolites of LAB is observed (Rathod et al., 2021). These metabolites include organic acids which help to reduce the pH of commodities which results in decreased cell membrane permeability. This results in the destruction of pathogenic cell membrane. In addition, nisin is a well-known LAB postbiotic metabolite that is potentially used for aquatic food preservation.

Lactic acid bacteria or LAB causes fermentation of carbohydrates and produces acids or other components in addition to lactic acid. As a biopreservative agent, the bacteriocins produced by these bacteria have broad spectrum activity against many pathogens (Singh, 2018). Nisin is a commonly known GRAS graded bacteriocin that is used in food products as it suppresses *Clostridium tyrobutyricum* in cheese. In acid-coagulated cheese types, these bacteriocins are added to inhibit *S. aureus*. Some bacteriocins, from postbiotics of heterofermentative bacterial species of *Leuconostoc citreum* MB-1 showed inhibition of *L. monocytogenes* ATCC-15313 in milk. This phenomenon occurs due to the presence of bacteriocin deemed as leucocin A – a heat-stable proteinaceous structure which is produced by most species of Leuconostoc bacteria. Shelf-life analysis showed that presence of this bacteriocin also delayed the pathogen growth at refrigerated temperature (Pujato, del L Quiberoni, Candioti, Reinheimer, & Guglielmotti, 2014).

2.6.2 Structurally defined postbiotics

2.6.2.1 Proteins

In dairy, LAB perform a specific role of breaking milk proteins into smaller peptides and generating smaller protein components and fragments. These peptides play a significant role in combating various diseases like various metabolic syndromes. In food products, these might act as antimicrobial and antioxidant agents.

1) Peptides: The number of peptides formed during fermentation depends upon the method employed for fermentation. The storage of fermented yogurt for at least 14 days increases the peptide quantity in samples (Nielsen, Jakobsen, Geiker, & Bertram, 2022). In the same way, the comparative study between normal fermented yogurt and heat-inactivated yogurt showed that the number of peptides produced in non-heat-treated yogurt is higher but both types produced distinctly different peptides. These are made up of various number of amino acids ranging from 2-20. The number and type of amino acids is also essential to identify the functional properties of various peptides (Ali et al., 2022).

<u>Mechanism of action</u>

- Mainly target the cytoplasm and form hole from which internal matter leaks
- Induces hydrolyses thus effecting cell’s out walls
- Cause acidity in cell membrane of pathogens (Aghebati-Maleki et al., 2021)

2) Bacteriocins and bacteriophages: Bacteriocins are polypeptides produced from ribosomes that are known to destroy pathogen cell by forming pores and inhibiting the synthesis of peptidoglycan from which bacterial cell wall is made. Their antimicrobial properties made them an interesting choice to be added as bioprotective and biopreservative agents in dairy fermented commodities.

Some bacteriocins, extracted from raw milk cheeses were studied for their activity against pathogens (Cavicchioli, Camargo, Todorov, & Nero, 2017). These bacteriocins are obtained from LAB strains of *E. hirae* (ST 57ACC) and *Pediococcus pentosaceus* (ST 65ACC). Mainly regarded as stable against sterilization temperature and resistant against Tween 80, the supernatants containing them significantly decreased the concentration of *L. monocytogenes* and *L. innocua*. Bacteriocins obtained were resistant against lipase and ɑ-amylase enzyme activity. After treating the postbiotics or cell free supernatants of *Enterococcus hirae*, with *L. monocytogenes* (strain 211 and 422), the growth was completely inhibited after 12 hours whereas the *P. pentosaceus* activity against these pathogens was limited due to unknown factors. As some species including *E. faecalis* and *E. faecium* are already part of various fermented products, it is deemed that *E. hirae* could also be potentially added in dairy commodities in future studies.

Bacteriocins from *L. acidophilus* (ATCC 4356) acted in a positive way to inhibit *Salmonella typhi*. *Salmonella typhi* causes typhoid when present in host digestive tract and often becomes resistant when treated with antibiotics in a prolonged way. (Pratiwi, Aditya, & Abbas, 2021). Hence, a solution to treat it naturally without antibiotic drugs could be achieved with the help of postbiotics. Since, *L. acidophilus* produces bacteriocins but according to the above study, these are produced at certain temperatures since their peptide nature makes them sensitive to temperature. Bacteriophages are added to certain dairy processed to maintain the freshness of these products. Bacteriophages help in reducing Salmonella in cheddar while it also helps to inhibit *S. aureus* in curd. In infant formula milks these cause inhibition of *Enterobacter sakazakii* (Singh, 2018).

3) Amino acids including GABA: GABA is also known as ɣ-aminobutyric acid. It is a class on non-protein amino acids. GABA is a functional food component that can be added to various foods in the form of additives. GABA is produced by α-decarboxylation of glutamate and this step is carried out by the enzyme glutamate decarboxylase. Since they are considered as Generally Recognized as Safe (GRAS) hence, it is deemed that their addition doesn't cause any harmful effect. In fact, these are known to regulate physiological behavior and are inhibitory neurotransmitters.

Some LAB obtained from kimchi are regarded to produce higher concentrations of GABA. Among the identified species, *Lb. brevis* strains are observed to be higher producer of GABA. The strains were identified via reversed phase HPLC instead of other chromatographical techniques (Wu & Shah, 2015). Cell free extracts of probiotic *Lb. plantarum* (strain DM5) produces significant amounts of GABA. The TLC assay confirmed that the strain produces significant quantities of GABA during incubation at 30 ºC for 30 hours. The results also showed that GABA spot was more obvious as the concentration of MSG was increased from 0.2 – 1.5 % which shows the activity of glutamate decarboxylate enzyme for the production of GABA (Das & Goyal, 2015).

2.6.2.2 Organic acids

LAB produces various organic acids during fermentation process. While it depends upon the type of fermentation that these bacteria carry out, lactic acid is common in both homo-fermentation and hetero-fermentation. In the later type, other organic acids like acetic acid, citric acid, succinic acid, etc. are also formed. Postbiotics in the form of organic acids are considered to act as antimicrobial metabolites against pathogens.

The antimicrobial effect cause by various strains of *Lb. Plantarum* is observed due to organic acids (Hu, Ren, Zhou, & Ye, 2019). This activity was seen to be more effective against Salmonella and *E. coli* and was deemed to be carried out due to lowering pH and acidification of bacterial cell-wall. Some literature also showed that some enzymes in the supernatant are responsible for production components that have antimicrobial effect. Enzymes in the postbiotics of *Pseudomonas aeruginosa* convert oleic acid to an effective antimicrobial compound named as DOD (7,10-dihydroxy-8 (E) -octadecenoic acid) (Tran et al., 2021).

Mechanism of action of organic acids

- Decrease the pH of the environment
- Solubilize the membrane components causing leakage of internal cellular components
- Decrease the cell permeability
- Inhibit enzyme activity when gain entry to the inside of pathogen cell
- Cause the acidification hence rupturing the cells (Rathod et al., 2021)

2.6.2.3 Short chain fatty acids

SCFAs are sometimes present in the outer wall of bacteria while in other cases these are excreted by the bacterial cells into the environment. SCFAs prominently includes butyric acid, acetic acid, propionic acid, etc. Butyrate is known for its activity against certain food allergies. When comes in contact with the targeted antigens, it causes unresponsiveness against them, hence, protects against them. Also, it produces IL-10 and T-reg that inhibit allergic reactions (Homayouni Rad, Aghebati Maleki, Samadi Kafil, & Abbasi, 2021).

Mechanism of action

- Increase membrane permeability of pathogen bacteria
- Cause variations and later disrupting the electron transport chain
- Cause functional changes in the pathogen bacteria
- Lysing the cell wall of pathogen bacteria (Higashi, Mariano, de Abreu Filho, Gonçalves, & de Oliveira, 2020)

2.6.2.4 Carbohydrates

Carbohydrate in the form of teichoic acid, oligosaccharides, polysaccharides etc. are mostly present in the outer bacterial cell wall. These components are important in many processed foods since they enhance not only flavor, mouthfeel, and taste but also increase the texture, stability, thickening and binging ability of food product. An innovative way of incorporating postbiotics is in the form of exopolysaccharides obtained from Lactobacillus bacteria. The process is developed to obtain exopolysaccharides zinc nanoparticles with antimicrobial properties that could be further used in the form of active packaging material (Krithika & Balasasirekha, 2021).

2.6.2.5 Some novel metabolites potentially used as postbiotics

Table 2.1 Some novel postbiotic components

Bacterial Source	Metabolite	Function	Reference
Limosilactobacillus reuteri	Reuterin and Reutericyclin	Cause oxidative stress in pathogenic cells, bactericidal effect against gram positive bacteria	(Gänzle, 2018)
LAB species	Sonorensin	Against gram positive and negative bacteria, inhibit biofilm production of S. aureus, enhanced membrane permeability	(Singh, 2018)

2.7 Novel Technologies Applied to Preserve the Activity of Postbiotics

2.7.1 Direct addition of lyophilized postbiotics

Lyophilization is also sometimes referred to as freeze drying and is a relatively common concept that involves the conversion of liquid phase to solid phase by applying extremely low temperatures with pressure. The process is safe to keep the metabolite profile of the postbiotic intact. Lyophilization of postbiotics from *Lb. acidophilus* LA-5, *L. salivarius* and *L. casei* at -40 ºC and applying 100 nTorr of pump pressure delivered lyophilized postbiotics that managed to maintain adequate amount of peptides, organic acids, alcohols, esters and fatty acids etc. Later investigation showed that these postbiotics from

L. salivarius were highly effective against biofilm removal of *L. monocytogenes*. Hence, the use of lyophilized postbiotics from above mentioned bacteria had the ability to inflict antibacterial activity in milk products (Moradi, Mardani, & Tajik, 2019).

2.7.2 Microencapsulation of postbiotics

The effectiveness of some metabolites produced by beneficial bacteria like lactic acid bacteria, is effected under certain conditions like high temperature, changes in pH, variation in pressure etc. To counter this situation, microencapsulation of postbiotics or sensitive metabolites (like short chain fatty acids) is done so that their activity remains intact. Many components like lipids, proteins, alginate, gelatin etc. could be utilized for microencapsulation (Rad et al., 2021).

One such example is the liposome microencapsulation od nisin-Z and its later addition in cheddar cheese (Benech, Kheadr, Lacroix, & Fliss, 2003). The resultant experimented products had antimicrobial activity and was also sensorily accepted and showed excellent flavor intensity. It is observed that 1.5 g/ 100 g was an optimal concentration that could be used in fermented beverage that doesn’t cause variations in its microstructure (Mora et al., 2019). A similar study on meat showed the intact antimicrobial activity of bacteriocin of *Lb. plantarum* due to microencapsulation with a combination of alginate-gelatin (Le et al., 2019).

3. MATERIALS & METHODS

All LAB species were obtained from the Department of Dairy Technology, Ankara University, Ankara. The experimental design was based on obtaining bacterial metabolites/ postbiotics from 10 LAB species, in the form of Cell Free Supernatants.

3.1 Bacterial Cultures Used in the Experiment

Ten different LAB bacteria were used to obtain metabolites. These bacteria included;

1) *Lactococcus lactis* subsp. *lactis*
2) *Enterococcus faecium*
3) *Enterococcus faecalis*
4) *Streptococcus thermophilus* 1-K_4
5) *Lactobacillus plantarum*
6) *Lactobacillus paraplantarum*
7) *Lactobacillus brevis*
8) *Lactobacillus helveticus* 9-B_5
9) *Lactobacillus bulgaricus* (Lb. 23)
10) *Lactobacillus coryniformis*

3.2 Activation of LAB Strains

100 µL of LAB stock (approx. 10^9 CFU/ mL) stored at -80 °C was inoculated into 5 mL of MRS and M17 liquid medium depending on the strain type. For lactobacilli MRS broth and for lactococci, streptococci and enterococci, M17 broth was used, respectively. Lactobacilli strains were incubated at 37 °C for 48 hours (under anaerobic conditions by keeping in air-tight jar with kit (Microbiology Anaerocult® A, Merck). Lactococci, Streptococci and Enterococci were aerobically incubated for 24 hours at 30 °C. The purity of the generated cultures was checked via the streak plate method on the related agar medium.

3.3 Determination of Bacterial Growth

The bacterial cycle comprises of 4 stages i.e., Lag phase, Log phase, Stationary phase and Death phase. Bacteria show exponential growth activity in Log phase and maximum number of metabolites are formed during. In the stationary phase maximum metabolites are produced as the bacteria is thriving to survive before the death phase. Identifying this period is critical in order to obtain postbiotics with maximum metabolites. To observe this growth pattern, optical density (OD) was checked by using spectrophotometer.

100 mL of M17 and MRS broths were separately prepared for each bacterial species. Initially, the OD for M17 and MRS broth was taken without inoculation, as the blank values. Then, *Lc. lactis*, *E. faecium*, *E. faecalis* and *S. thermophilus* 1-K_4 were separately inoculated in M17 broth and the optical density was immediately taken before putting them in incubator. After that broth containing these bacteria were put in incubator at 30 ºC. The same process was carried out for *Lb. plantarum*, *Lb. paraplantarum*, *Lb. brevis*, *Lb. coryniformis*, *Lb. bulgaricus* and *Lb. helveticus* 9-B_5. These were separately inoculated in MRS broth and readings for OD were taken for each bacterium according to the same procedure during the anaerobic incubation at 37 ºC. After every 2 hours, approximately 2-3 mL of each sample was taken carefully in the quvets and was measured. The process was carried out for 24 hours. for Lactococcus, Enterococcus and Streptococcus and till 2 days for Lactobacillus species. The end of logarithmic phase obtained via OD determination in which the bacterial metabolites are found at most was chosen as the incubation period for each strain (Table 4.1).

3.4 Obtaining Postbiotics

Broths with bacterial culture were taken out from the incubator after incubation time was over. pH was checked for all bacterial species and recorded. At this point, broth contained both live bacteria and their metabolites - also known as Supernatant or Postbiotics. The grown cultures in broths were centrifuged at 8000 rpm for 5 min at 4 ºC to separate pellets and the supernatants. The cell-free supernatants were taken in another sterilized falcon tube and was kept at -80 ºC for further analysis.

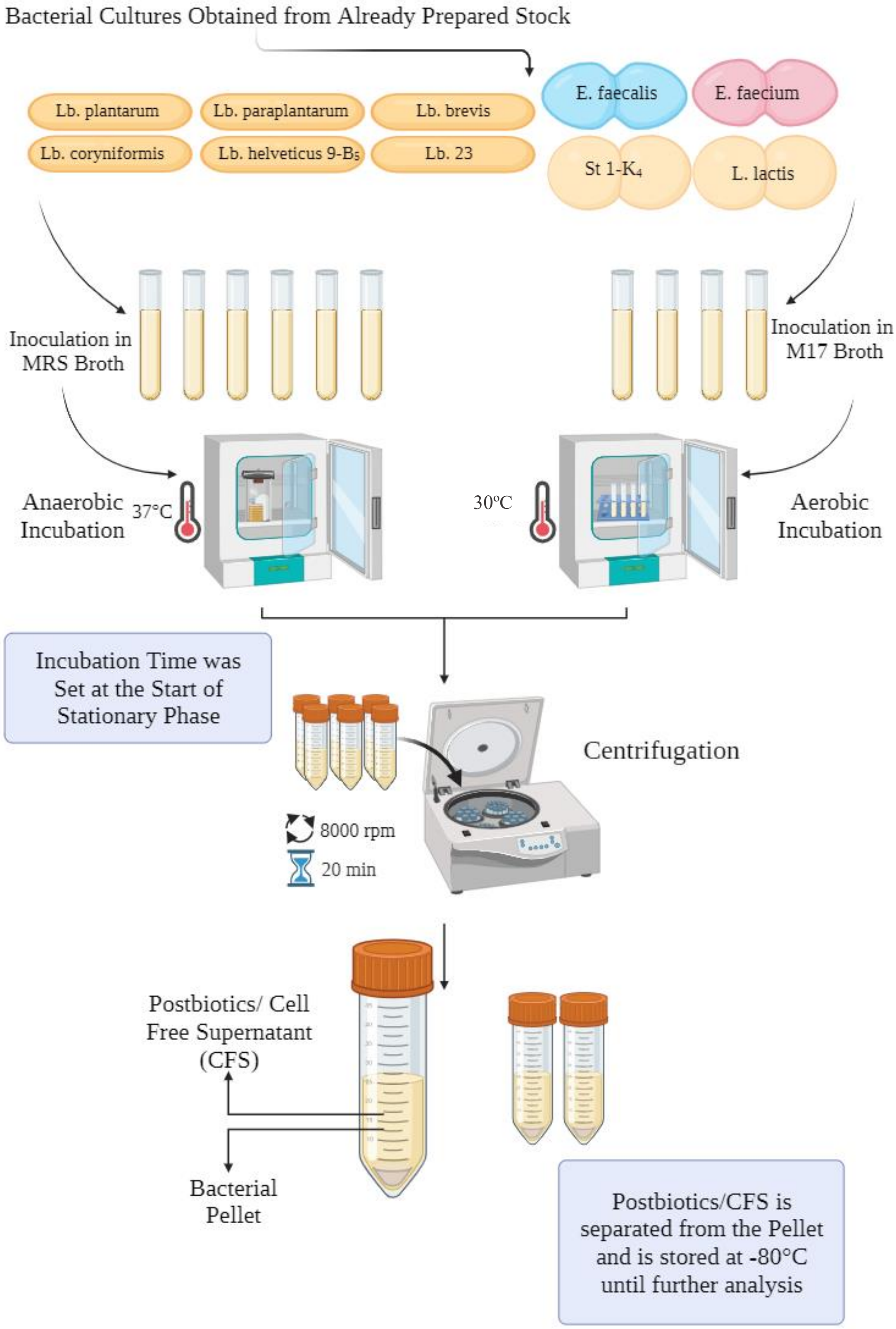

Figure 3.1 Process for the postbiotics preparation

3.5 Functional Analysis for Postbiotics

Postbiotics obtained from 10 different bacterial strains were analyzed for their potential antioxidant activities, anti-microbial activities, phenolic contents, and organic acid contents.

3.5.1 Presence of phenolic contents

Phenolic components are secondary compounds and are identified by the presence of -OH group attachment to the aromatic benzene ring. These phenolic contents are utilized for various antioxidants, anti-inflammatory, and as a natural defense system in the commodity against potentially harmful foreign factors. LAB naturally produces metabolites with significant phenolic contents. These were calculated by comparing with standard gallic acid. For this aim, 5 mg/ mL gallic acid was prepared as main stock solution and the diluted standard solutions were prepared at the concentrations of 4, 3, 2, 1, 0.75, 0.5, 0.25, 0.10, 0.05 mg/ mL. Measurements were performed with spectrophotometer at 760 nm and the graphic curve was formed (Appendix 2).

During the phenolic content analyses, 100 µL postbiotic sample was added 500 µL Folin-ciocalteu reagent, 1.5 mL of 20 % Na_2CO_3 and 7.9 mL of distilled water in a test tube. The mixtures were vortexed and kept in dark place at room temperature for an incubation period of 1 hour. After this duration, the absorbances were read at 760 nm in the spectrophotometer twice, and average was taken. The data were obtained by comparison with the gallic acid equivalence formula given below in which the 'y' is the average absorbance calculated for each postbiotic and 'x' is the concentration of phenolic content compared to gallic acid. The values were obtained with the unit of mg GAE/mL.

$$y = 1.3356x - 0.0413$$

$$x = \frac{y + 0.0413}{1.3356}$$

3.5.2 Determination of antioxidant properties

Since postbiotics are deemed to be enriched with beneficial bacterial metabolites having significant radical scavenging properties, they are analyzed for their antioxidant properties via DPPH assay method. The antioxidant activity of all the postbiotics were compared with Trolox standard which has excellent antioxidant properties. DPPH solution was prepared in 0.1 mM molarity in methanol (MeOH) and kept in a refrigerator at 4 ºC in the dark. The solution could be used for 2-3 days in general but for each analysis freshly prepared DPPH solution was used.

10mM of main trolox standard stock solution was prepared in ethanol. Dilutions were made from this solution to obtain the standard curve at the concentrations of 5, 2.5, 1, 0.25, 0.1, and 0.05 mM in distilled water (Appendix 1). The absorbance of all standards was read in the spectrophotometer twice.

To perform antioxidant analyses via DPPH assay, 3 triplicates were analyzed for each postbiotic. 100 µL of sample was added into 2 mL of prepared DPPH solution. As blank MRS and M17 broths were used instead of sample. All samples were kept in the dark and at the refrigeration temperature of 4 ºC. The incubation time was 30 min. After 30 min, absorbance of all samples was checked in spectrometer at 517 nm wavelength. The inhibition % was estimated by using the following formula;

$$\%\ \text{Inhibition} = \frac{A_{\text{Blank-}} - A_{\text{Sample}}}{A_{\text{Blank}}} \times 100$$

A_{blank}: Absorbance of DPPH solution with water instead of sample.
A_{sample}: Absorbance of DPPH solution with sample.

By identifying the inhibition % for each postbiotic, values were compared with the trolox standard. The formula was extracted from the trolox standard curve (Appendix 1) that was carved by using different concentrations of trolox. The values were calculated in mM

TEAC. Antioxidant activity of each postbiotic was calculated by using following formula;

$$y = 17.465x + 0.2188$$

$$x = \frac{y - 0.2188}{17.465}$$

3.5.3 Antimicrobial analyses

The antimicrobial properties of prepared postbiotics were tested separately against 6 pathogens (*Staphylococcus aureus* (ATCC 25923), *Mycobacterium tuberculosis* (ATCC 7644), *Escherichia coli* (ATCC 25922), *Bacillus cereus* (ATCC 14579), *Salmonella enterocolitica* (ATCC 14028), *Enterococcus faecalis* (ATCC 29212) via the agar well diffusion method. The selective medium of Brain Heart Infusion (BHI) was used for the analyses.

The stock cultures of above-mentioned pathogens (~10^8 log CFU/ mL) kept at -80 ºC were used for the activation. 100 µL of each pathogen stock culture were inoculated in 5 mL of BHI broth, individually, and incubation was performed at 37 ºC for 18-24 hours. Dilutions were also prepared in a ratio of 1:9. The petri plates including BHIA were taken, and each was virtually divided into 4 sections (by drawing lines on the bottom of the plate). Later, wells were formed in each section with a sterile cork-borer. During analyses, for each plate, 100 µL of activated pathogen was spread onto the plate from diluted concentration of 10^6 CFU/mL. For testing against six pathogens, 50 µL of postbiotic obtained from each LAB was introduced in the designated well marked for that specific postbiotic. In each plate, one well was prepared with distilled water as control. The plates were incubated at 37 ºC in an incubator for 24 hours. The antimicrobial activity was observed by checking the clear zone around each well.

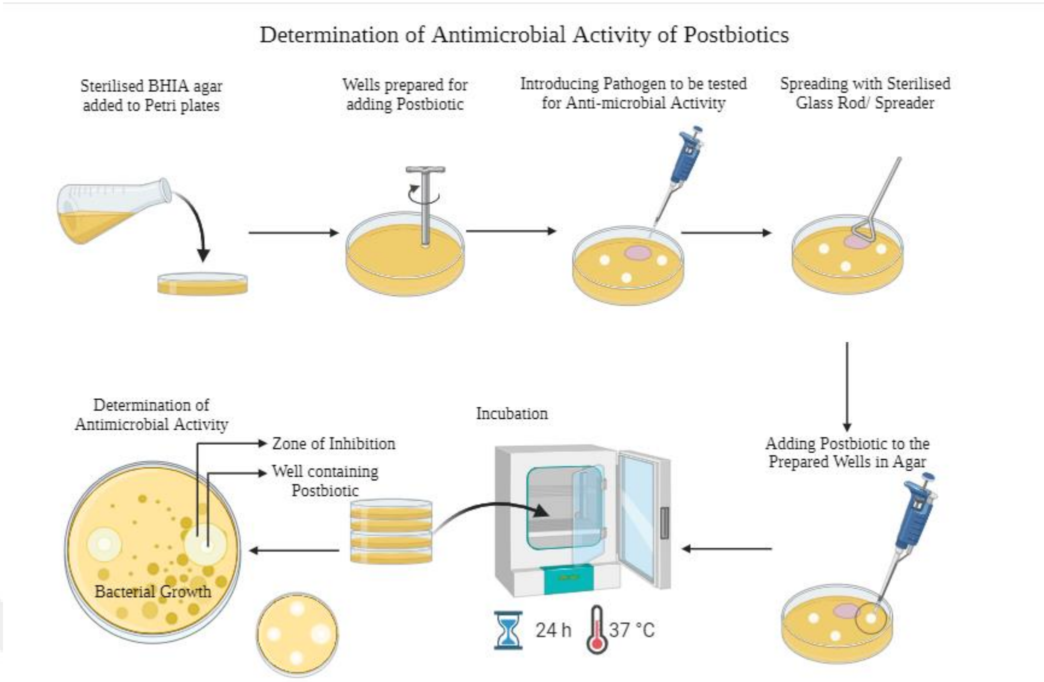

Figure 3.2 Agar well diffusion method to check anti-microbial activity of postbiotics.

3.5.4 Organic acid profile

The organic acids present in each postbiotic were determined via HPLC system. 0.013N H_2SO_4 was used as mobile phase. The HPLC column used for the analysis was a MetaCarb 87H column (300 x 7.8 mm) from Agilent Technologies. The flow rate was set as 0.9 mL/min and column temperature was set at 65 ºC. The analysis period for each postbiotic was approximately 30 min (Bulat & Topcu, 2020). Citric, formic, pyruvic, succinic, lactic, propionic, acetic, and butyric acids were detected at a wavelength of 210 nm while acetoin and diacetyl were detected at 280 nm by using UV detector.

Postbiotic samples were prepared by adding 5 mL of individual postbiotic to 1 mL 0.013N H_2SO_4. The mixtures were vortexed for 2 min. Prepared samples were filtered via passing through a 0.22 µm syringe filter before introducing to the HPLC system. 20 µL postbiotic sample was injected for the measurement. Peak areas were obtained for the calculation of the concentration of the organic acid present in the sample. Calculation of all organic acids, acetoin, and diacetyl concentration in each postbiotic sample was done according to standard curves obtained for each respective compound.

Analytical standard of each organic acid was prepared in 3200 ppm (mg/ L) as stock solution and the working standard solutions were prepared at the concentrations of 1600, 800, 400, 200, 100, 50 ppm. Measurements were obtained with the same analysis conditions as the samples. Standard calibration curves were plotted for each organic acids, acetoin, and diacetyl.

3.6 Determination of Bio-preservatory Role of Postbiotics in Pasteurized Milk

100 mL sterile glass jars were used for bio-preservatory analysis. Pasteurized milk (72 ºC for 2 min) was added into jars. Later, 200 μL of postbiotic belongs to each LAB was incorporated to pasteurized milk and stirred gently for a homogeneous mixture. Control sample was obtained as 100 mL of pasteurized milk without any postbiotic. The jars were completely closed and were placed in the refrigerator at 4 ºC for 10 days. Below mentioned analyses were performed during storage on day 1, 5 and 10.

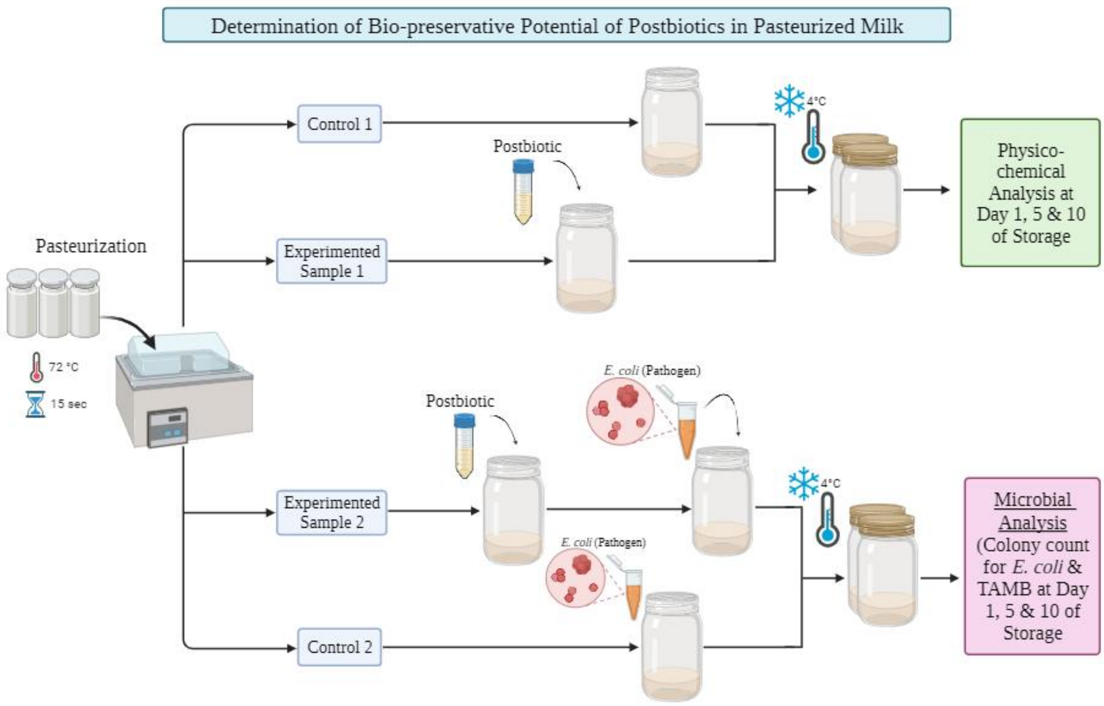

Figure 3.3 Experimental design for the bio-preservation analyses.

3.6.1 Physicochemical analysis of pasteurized milk during storage

3.6.1.1 pH

pH was measured with a pH meter (Starter300 pH meter, Ohaus).

3.6.1.2 Titratable acidity (Lactic acid %)

Titratable acidity was performed according to AOAC (1995). During analyses, 10 g sample was taken, and 2-3 drops of phenolphthalein indicator were added. Titration was performed by using 0.1N NaOH until light pink hue was observed. The consumed quantity of NaOH was used for the calculation according to the below formula.

$$\text{Lactic acid (\%)} = \frac{\text{V} \times 0.009}{\text{g}} \times 100$$

where;
V = quantity of 0.1 N NaOH used (mL)
g = quantity of milk

3.6.1.3 Total solid (%)

The total solid % of milk samples were analyzed by the gravimetric method at 102 °C (IDF, 1982) and calculated according to the below formula:

$$\text{Total solid \%} = \frac{W_3 - W_1}{W_2 - W_1} \times 100$$

Here;
W_1 = Weight of empty container (g)
W_2 = Weight of sample and container (g)
W_3 = Weight of dry sample and container (g)

3.6.1.4 Determination of rheological properties

Rheology analyses were performed using Kinexus Pro+ rheometer (Malvern Panalytical, Malvern, UK) attached with a 40 mm stainless steel 4º conical geometry spindle and with 1mm gap. Temperatures of frequency sweep measurements were set at 5 °C. The shear rate was set between 0.1 – 300 s^{-1}. The flow curves were fitted to the Power law model by using non-linear regression. The Power law model equation is stated as below:

$$\sigma = K.\gamma^{n}$$

where; σ is shear stress (Pa), K is a consistency index (Pa.s), γ is the shear rate (1/s), and n is the flow behavior index.

3.6.1.5 Color determination

Color determination for all samples was done at the end of 10-day storage. Color data was determined in CIE-Lab. Color Spectro-colorimeter (Portable Color Spectrocolorometer, Model SL400, ser-lab) used for this purpose was first calibrated using white and black background as indicated in the instructions. After that, milk sample was added to the container and readings were taken for L* (degree of lightness), a* (degree of redness) and b* (degree of yellowness).

3.6.2 Estimation of bio-preservation capacity of postbiotics

According to the results of antimicrobial activity, most postbiotics showed potential antimicrobial activity against *E. coli* (ATCC 25922) while some also showed antimicrobial activity against other pathogen. Hence, *E. coli* was selected to observe the antimicrobial potential in model pasteurized milk sample containing postbiotics.

3.6.2.1 *E. coli* count

For estimating *E. coli* count, serial dilutions (10^{-4}, 10^{-5} and 10^{-6}) were made, and 100 µL of these diluted samples were evenly spread on the BHIA medium by using spread plate method. The petri plates were kept for incubation at 37 ºC for 24 hours. On the next day pathogen colonies were counted and a log reduction was noted. The process was repeated for all samples and was carried out at day 1, 5 and 10 of storage period. Two controls were also prepared for comparing the results. In the first control, only pasteurized milk was taken while in the second, only pathogen *E. coli* (100 µL) was added to the pasteurized milk without any postbiotic. The controls were also incubated and stored under the above-mentioned conditions.

3.6.2.2 Total aerobic mesophilic bacteria count (TAMB)

Plate Count Agar (PCA) was used for the enumeration of TAMB. For this purpose, 100 µL of diluted samples (having both *E. coli* (ATCC 25922) and postbiotic in pasteurized milk) was added and spread using spread plate method. The petri plates were incubated at 37 ºC for 24 hours.

4. RESULTS AND DISCUSSION

4.1 Incubation Time For Each Bacterium

The OD (optical densities) of growth medium with bacteria were observed every 2 hours to identify the beginning of stationary phase of each bacterium. Identifying the beginning of stationary phase was essential because at this phase bacteria can produce maximum metabolites and the growth medium might also have other metabolites than the functional ones. Analyses of the optical densities provided the information regarding the incubation time to be given to each bacterium in order to obtain maximum target postbiotics without the unwanted metabolites.

Table 4.1 Beginning of stationary phase of LAB strain determined via OD measurements

Sample	**Time of beginning of stationary phase (hours)**
E. faecalis	24h
E. faecium	22h
St. thermophilus 1-K_4	18h
Lc. lactis	24h
Lb. plantarum	20h
Lb. paraplantarum	22h
Lb. brevis	24h
Lb. coryniformis	36h
Lb. helveticus 9-B_5	36h
Lb. bulgaricus	36h

4.2 Functional Properties of Postbiotics

4.2.1 Phenolic contents in postbiotics

Phenolic contents are the secondary metabolites that are identified with the presence of at least a single hydroxyl group attached to the benzene ring. These contents have direct effect on the casein protein aggregation and add flavor to some cheese (O'connell & Fox, 2001). In milk, they are deemed to hinder the color development due to Millard reaction that mostly occurs due to high temperature treatment (Kokkinidou & Peterson, 2014).

Table 4.2 Phenolic contents of the postbiotic of the relevant strains

Sample	Phenolic content (mg GAE/mL)
E. faecalis	0.66 ± 0.04^{ab}
E. faecium	0.54 ± 0.01^{b}
St. thermophilus **1-K$_4$**	0.55 ± 0.00^{b}
Lc. lactis	0.63 ± 0.06^{ab}
Lb. plantarum	0.61 ± 0.03^{ab}
Lb. paraplantarum	0.72 ± 0.03^{a}
Lb. brevis	0.55 ± 0.06^{b}
Lb. coryniformis	0.64 ± 0.08^{ab}
Lb. helveticus **9-B$_5$**	0.66 ± 0.00^{ab}
Lb. bulgaricus	0.60 ± 0.00^{ab}

The differences ($P < 0.05$) among all samples were indicated with lower-case letters (a, b, ab etc.). Values are shown as Mean ± SD.

According to the above data, postbiotics from *Lb. paraplantarum* showed maximum amount of phenolic contents (0.72 mg GAE/mL) as compared to other postbiotics. Phenolic contents in postbiotics of *Lb. plantarum* were also high (0.61 mg GAE/mL). These findings showed similarity to the observations of Nazareth et al. (2023) who were able to find significant amount of numerous phenolic contents from postbiotics of *Lb. plantarum*.

The main reason for high amount of phenolic content lies in the ability of LAB to get adapted to the native environment (Rodríguez et al., 2009). As these LAB were taken from the dairy source hence, it also predicts that metabolites from *Lb. plantarum* could perform quite well in dairy commodities. The high amount of phenolic contents detected in postbiotics of *Lb. plantarum* could also be directly linked with β-glucosidase enzyme which is present in the metabolites of *Lb. plantarum* which hydrolyses the flavonoid compounds and increase the bioavailability of phenolic compounds (Degrain, Manhivi, Remize, Garcia, & Sivakumar, 2020).

According to Antognoni et al. (2019), not all strains of *Lb. plantarum* were able to produce phenolic compounds as only three out of total eight *Lb. plantarum* strains including *Lb. plantarum* 98A, *Lb. plantarum* 29DAN and *Lb. plantarum* LB126, were able to produce some phenolic contents. Adequate amounts of phenolic contents were detected from postbiotics of *E. faecalis*, *Lb. plantarum* and *Lb.* helveticus 9-B_5. Li et al. (2021) also confirmed production of high amounts of phenolic contents by LAB species of *Lb. plantarum* and *Lb. helveticus* of 2 g GAE/L and 2.2 g GAE/L, respectively. The high content was due to the presence of viable bacteria which continued its activity during 48 hours of incubation.

Li et al. (2021) also suggested that high phenolic content could be contributed by the presence of hydrolytic enzymes which are secreted by LAB during incubation or might be released by deglycosylation of glycosylated phenols. Formation of phenolic content occur due to the presence of p-coumaric acid instead of ferulic acid (Couto, Campos, Figueiredo, & Hogg, 2006).

In this study, much higher phenolic compounds were observed from the postbiotics of *Lb. brevis* as compared to the findings of Sornsenee et al. (2021) where only > 0.25 mg GAE/ mL was observed. The results for the phenolic contents obtained from *Lb. bulgaricus* were similar to the results observed by Hamad et al. (2022).

4.2.2 Antioxidant activities of postbiotics

The antioxidant activity is important in inhibiting the bad flavors that develop in dairy over time. Pasteurized milk can stay fresh for up to 14 days but it's qualitative value decrease due to the oxidation of some components (Castillo, Pereira, Abuelo, & Hernández, 2013). In humans, antioxidant compounds shows inhibitory activities against many diseases including cancer. Heat killed cells of LAB including *Lb. plantarum, Lb. bulgaricus,* etc. effectively reduce the growth of breast adenocarcinoma cells in-vitro studies (Liu & Pan, 2010). Postbiotics from LAB showed abundant antioxidant properties. In the carried-out study, the antioxidant activities of all postbiotics were compared against Trolox standard.

Table 4.3 Antioxidant activity of the postbiotic of the relevant strains

Sample	**DPPH (TEAC mM/ mL)**
E. faecalis	3.60 ± 0.11^{a}
E. faecium	3.46 ± 0.16^{a}
***St. thermophilus* 1-K$_4$**	3.34 ± 0.07^{a}
Lc. lactis	3.58 ± 0.18^{a}
Lb. plantarum	3.67 ± 0.25^{a}
Lb. paraplantarum	3.08 ± 0.06^{ab}
Lb. brevis	3.24 ± 0.02^{a}
Lb. coryniformis	2.23 ± 0.33^{bc}
***Lb. helveticus* 9-B$_5$**	1.88 ± 0.00^{c}
Lb. bulgaricus	2.35 ± 0.46^{bc}

The difference ($P < 0.05$) among all samples was indicated with lower-case letters (a, b, ab etc.).

According to the obtained results, postbiotics from *Lb. plantarum* showed highest antioxidant activity among all the tested postbiotics while the lowest antioxidant activity was observed by the postbiotics of *Lb. helveticus* 9-B$_5$. Postbiotics from Lactobacillus species showed lower amounts of antioxidant activity while that from Enterococcus species were significantly high. High phenolic content detected from the postbiotics of *E. faecalis* (shown in Table 4.1) could be responsible for showing high antioxidant activities.

Antioxidant activities were also found in the postbiotics obtained from *St. thermophilus*. The radical scavenging activities of postbiotics of *St. thermophilus* in our findings were also found to be similar to the results of Wang & Li (2022). Also, much similar inhibition percentages were observed for postbiotics which became possible due to the same incubation time (18h) that was given to the bacteria and same type of postbiotics were obtained. Additionally, *Lc. lactis* also showed comparatively higher antioxidant activity of 3.58. High antioxidant activity of cell free supernatant of *Lc. lactis* was also observed by De Marco et al. (2018).

High antioxidant properties were observed from postbiotics of *Lb. plantarum* which were much higher compared to the findings of (Noori et al., 2023). Similarly, high antioxidant properties of postbiotics were observed from *Lb. plantarum* NJAU -01 (Liu et al., 2021), *Lb. plantarum* strain Lp-790, and Lp998 (Zago et al., 2017) and from postbiotics of *Lb. rhamnosus* (Rajoka et al., 2019). High antioxidant activity of postbiotics of *Lb. plantarum* could also be linked with the presence of high phenolic contents. In a similar way, we can observe the higher DPPH radical scavenging activity of CFS of *Lb. plantarum* and *Lb. brevis*, from the findings of (Vougiouklaki et al., 2023). Here the main reason lies in the time duration that was initially provided for incubation. The average incubation time that we gave was 20h and 24h for *Lb. plantarum* and *Lb. brevis* respectively while in above mentioned study the incubation time extremely higher of around 5 days.

In terms of postbiotics taken from *Lb. bulgaricus*, the antioxidant activity was not significantly high as compared to that of other postbiotics. The reason might be the concentration of postbiotics that was used. Inhibition (%) using DPPH deviates with the change in concentration of postbiotic (Hamad et al., 2022). Although the inhibition % of *Lb. bulgaricus* was lower in our study, it was found to be higher when compared to the findings of Lin & Yen (1999). According to Lin & Yen (1999), intracellular cell free extract of all 6 strains of *Lb. bulgaricus* (448, 449, 1006 etc.) showed significantly higher inhibition percentage. The reason could be the type of postbiotic that was used. Intracellular cellular free extracts might exhibit lesser antioxidant activity as compared to the cell secretions or intercellular metabolites. The same author showed similar results for *St. thermophilus* strains as well.

The results for postbiotics of *Lb. bulgaricus* were also contradictory to the ones obtained by Liu & Pan (2010), who observed higher antioxidant potential of postbiotics of *Lb. bulgaricus* BCRC 10696 (extracted from Bulgarian yogurt). The reason could be the method that was employed to obtain these postbiotics, as in above-mentioned the heat-killed cells (121 ºC) and their cytoplasmic cell fractions were taken as postbiotics instead of metabolites secreted by bacterial cells. In case of postbiotics of *Lb. helveticus* 9-B_5 and *Lb. bulgaricus*, although they possessed good amount of phenolic contents, these contents didn't exhibit good antioxidant activities.

4.2.3 Antimicrobial activity shown against various pathogens

Although there is little probability for the survival of pathogens after pasteurization, environmental contamination, and lack of hygiene could become a reason for the recontamination of milk even after pasteurization (Eneroth, Christiansson, Brendehaug, & Molin, 1998). In this case, postbiotics having antimicrobial activity could act to reduce pathogens and other spoilage microorganisms. To observe the antimicrobial activity of postbiotics from LAB species, each postbiotic was exposed to certain pathogens. The antimicrobial activity of all postbiotics observed is shown in Table 4.4.

According to the data, all postbiotics showed effective reduction of *E. coli* which was represented by at least 1 mm of inhibition zone around the well for postbiotics from *Lb. brevis*, *St. thermophilus* 1-K_4, *Lb. plantarum*, *Lb. paraplantarum* and *E. faecalis*. This similar but intense antimicrobial activity against *E. coli* was also observed by using cell free supernatant of *Lb. plantarum* strain isolated from kefir (Puertollano et al., 2009). The antimicrobial activity of cell-free supernatant of *Lb. plantarum* and *Lb. brevis* against *E. coli* were also evident in the findings of Yolmeh et al. (2017), but in that case, the mixture of postbiotics was used against *E. coli* so the antimicrobial effect shown by single postbiotic was difficult to find. A decrease in coliforms colonies is contributed by *Lb. plantarum* in some food commodities like olives (Marsilio et al., 2005).

In the opposite case, no anti-microbial activity was shown by any postbiotic against *B. cereus* and *S. aureus*. The minimum antimicrobial activity against *S. aureus* were contrary to the findings of Taj et al. (2022), who observed adequate antimicrobial activity against *S. aureus* by various *St. thermophilus* strains. This could be possible due to a variations among different *S. aureus* strains. Additionally, neither *St. thermophilus* nor *Lc. lactis* were able to produce any anti-microbial effect against *S. aureus* (Hladíková, Smetanková, Greif, & Greifová, 2012). In another study, postbiotics of *Lb. plantarum* NRRL B-4496 strain showed high antimicrobial activity against *S. aureus* (ATCC 25923) which was due to the secretion of different amounts of organic acids by strains of same bacteria (Arrioja-Bretón, Mani-López, Palou, & López-Malo, 2020).

This antimicrobial effect observed due to presence of organic acids is further confirmed by de Carvalho, et al. (2006), who observed the significant decrease in antimicrobial activity of cell-free supernatant of LAB when tested after its neutralization. Postbiotics from *E. faecalis* were significantly effective against *M. tuberculosis* and pathogen *E. faecalis* which was shown by the 2 mm inhibition zone. *Lc. lactis* postbiotics were also effective against *M. tuberculosis* as their activity was reduced up to 2mm around the well. Additionally, reduced activity of *S. enterecolitica* (ATCC 14028) was seen for postbiotics of *Lb. plantarum*, *Lb. paraplantarum*, and *Lb. coryniformis*.

In terms of postbiotics of *Lb. brevis*, some antimicrobial activity was detected against *E. coli* (ATCC 25922) which was thought to be contributed by citric acid and pyruvic acid which were detected in abundance in these postbiotics. Contrarily, this activity was not observed in the findings of Arrioja-Bretón et al. (2020), who reported no inhibition zone against cell-free supernatant of *E. coli* (ATCC 25922), which is due to different strain of *Lb. brevis* to obtain those cell-free supernatant.

Table 4.4 Antimicrobial activities of various postbiotics

Pathogen \ Sample		*Lb. bulgaricus*	*Lb. helveticus* 9-B_5	*Lb. coryniformis*	*Lb. brevis*	*Lb. paraplantarum*	*Lb. plantarum*	*Lc. lactis*	*St. thermophilus*	*E. faecium*	*E. faecalis*
E. coli **(ATCC 25922)**	R-1	positive	positive	positive	1mm	x	1mm	x	1mm	positive	1mm
	R-2	positive	x	positive	x	1mm	1mm	x	x	positive	x
E. faecalis **(ATCC 29212)**	R-1	x	x	x	x	x	x	x	x	x	x
	R-2	x	x	positive	x	1mm	x	2mm	x	x	2mm
B. cereus **(ATCC 14579)**	R-1	x	x	x	x	x	x	x	x	x	x
	R-2	x	x	x	x	x	x	x	x	x	x
S. enterocolitica **(ATCC 14028)**	R-1	x	x	1mm	x	1mm	2mm	x	x	x	x
	R-2	x	positive	x	x	x	x	x	x	x	x
S. aureus **(ATCC 25923)**	R-1	x	x	x	x	x	x	x	x	x	x
	R-2	x	x	x	x	x	x	x	x	x	x
M. tuberculosis **(ATCC 7644)**	R-1	x	x	x	x	x	x	1mm	x	x	x
	R-2	x	x	x	x	x	x	2mm	positive	1mm	2mm

Positive = minimum antimicrobial activity which is unmeasurable but detectable; x = no antimicrobial activity.

4.2.4 Organic acids, acetoin and diacetyl amounts detected from postbiotics

4.2.4.1 Citric acid

Citric acid is considered one of the weakest organic acids which is often used in the food industry as a mean of natural preservative. Citric acid produces an acidic environment when added in certain food commodities thus, hindering the survival of many pathogens (Hosseini, Abbasi, Sabahi, & Khani, 2021).

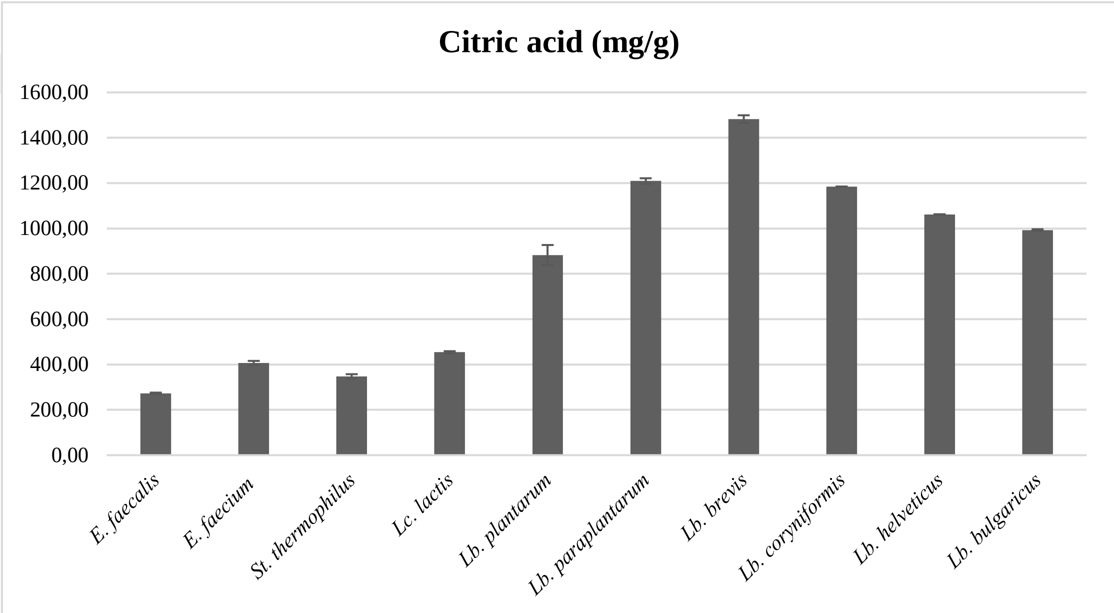

Figure 4.1 Citric acid concentration in each postbiotic

Compared to all other organic acids, the amount of citric acid detected in all postbiotics was lower. Among these postbiotics, *Lb. brevis* showed highest citric acid quantity ranging to as much as 1482.31 mg/g. Postbiotics produced by Enterococcus species (*E. faecium* and *E. faecalis*) showed the lower amounts as compared to others while minimum amount of 347.41 mg/g was shown by *St. thermophilus* 1-K_4. Some postbiotics extracted from *Lb. reuteri* also exhibits some amount of citric acid as cited by Jamaran et al. (2021), and these amounts are similar to our results.

Additionally, bacteria like *Aspergillus niger* and *Candida spp.* are known to be large producers of citric acid (Vandenberghe, Soccol, Pandey, & Lebeault, 1999). Since the experimented native LAB species are not considered to be commercial producers of citric acid, it was clearly seen that the amount produced by these bacteria was much lower.

Also, the amount of citric acid produced by these bacteria depends on the source and amount of sugar present in the growth medium as, lower amounts of lactose in M17 broth, which is also a weak sugar might be the reason for low production of citric acid (M. Hossain, Brooks, & Maddox, 1984). In a study done by Li et al. (2021), similar amounts of citric acid were observed for *Lb. helveticus* and *Lb. plantarum*, when inoculated with jujube juice (Hetian variety) for 48 hours at 37 ºC. Although a slight difference of organic acid is evident because of different incubation time and other conditions.

4.2.4.2 Pyruvic acid

Pyruvic acid is an α-keto acid which is considered as an important intermediate metabolite produced during the breakdown of carbohydrates by LAB. Some studies suggest its importance in the reduction of zinc toxicity in human brain cell that could lead to low-level brain injury (Maleki & Eiteman, 2017). Since chemical synthesis of pyruvic acid is expensive, microbial synthesis is considered as a potential way for its production.

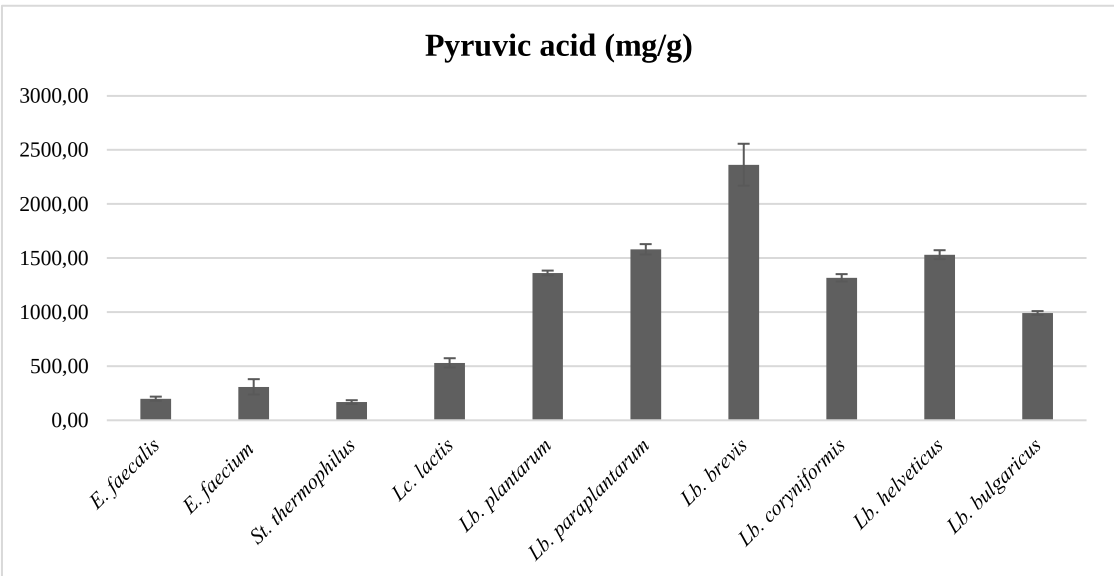

Figure 4.2 Pyruvic acid concentration in each postbiotic

Significantly high amounts of pyruvic acid were detected from postbiotics of *Lb. brevis* (2363.48 mg/g), followed by postbiotics of *Lb. paraplantarum* (1581.66 mg/g). Postbiotics from *St. thermophilus* 1-K_4 showed the lowest pyruvic acid amount of around 167.67 mg/g. High pyruvic acid production by *Lb. brevis* could be due to the presence of pyruvic acid kinase activity which is often secreted by *Lb. brevis* and cause the conversion of 3-phosphoglyceric acid to pyruvate (Eltz & Vandemark, 1960).

The lower amount of pyruvic acid produced by Enterococcus species could be explained by the absence of appropriate incubation conditions. Adequate production of pyruvic acid was achieved from *Enterococcus casseliflavus* A-12 under alkaline conditions and at a pH of 10 (Yanase et al., 1992). Some Streptococcus strains produce pyruvic acid as an intermediate acid which is either oxidized to acetic acid or is reduced to lactic acid (Pierce Jr, 1957). Hence, decreased amount of pyruvic acid detected by St. thermophilus could be due to its conversion into other organic acids.

4.2.4.3 Succinic acid

Succinic acid is commonly present in living species in the form of succinate. It has various benefits in pharmaceutical and food processing via adding sensory properties like flavor to certain food commodities (Ladnova et al., 2021). Some studies show that this acid has an inhibitory effect similar to acetic acid and could be effectively used as a biopreservative agent by acting as a buffer in pH balancing.

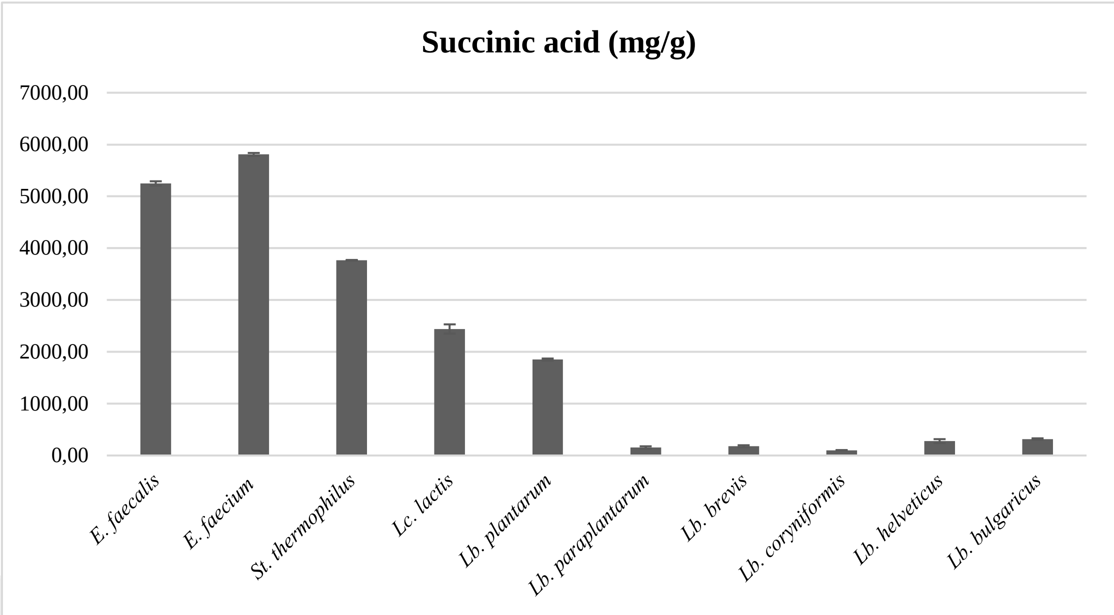

Figure 4.3 Succinic acid concentration in each postbiotic

The results of the postbiotics extracted from HPLC method showed that Enterococcus species produce the highest amount of succinic acid with *E. faecium* producing 5808.61 mg/g and *E. faecalis* potentially producing 5249.42 mg/g of succinic acid. The variation in the composition of growth medium plays a complex role in determining the amount of succinate and succinic acid produced by Enterococcus species, because the succinic acid production is largely enhanced by the presence of yeast extract in M17 broth medium (Kang, Yun, & Ryu, 2000).

The amount of succinic acid produced by *St. thermophilus* 1-K_4 and *Lc. lactis* was much higher than the findings of Özcelik et al. (2016), who revealed half the amount of succinic acid produced by *St. thermophilus* NCFB2392 in MRS broth but higher amounts in anchovy infusion broth which could occur due to difference in bacterial strains and their performance in across various growth mediums.

Lactobacillus species, including *Lb. brevis*, *Lb. paraplantarum*, and *Lb. coryniformis* produced the lowest amounts of succinic acid. Lactobacillus bacteria produce succinic acid by utilizing diammonium citrate present in the de Mann Rogosa-Sharpe growth media (MRS). The ability to utilize diammonium citrate is linked to the bacterial strain as not all lactobacillus bacterial strains have the ability to produce succinic (Kaneuchi, Seki, & Komagata, 1988). Also, LAB like *Lb. brevis* might only produce succinic acid via bioconversion of some acids like tartaric acid and the absence of this results in lower amount of succinic acid as described by (Radler & Yannissis, 1972).

The results for the postbiotics of all lactobacillus species shows that these species have minimum ability to produce succinic acid. This same lack of ability to produce succinic acid was also observed from the postbiotics of another lactobacillus specie i.e., *Lb. reuteri* (Jamaran, Jafari, Marjani, Akbari, & Feizabad, 2021). Similarly, in another study by Rozhkova et al. (2023), minimum quantities of succinic acid were found when compared to the quantities of other organic acids which also proves our findings.

4.2.4.4 Lactic acid

Lactic acid is considered as one of the most important organic acid that is mainly produced by LAB and which commonly functions as a bio preservative agent in most dairy products via fermentation (Stiles, 1996). Although lactic acid has limited utility in pasteurized milk, but its addition is deemed to be essential in enhancing shelf life by inhibiting the growth of pathogenic bacteria (Hathout & Aly, 2010).

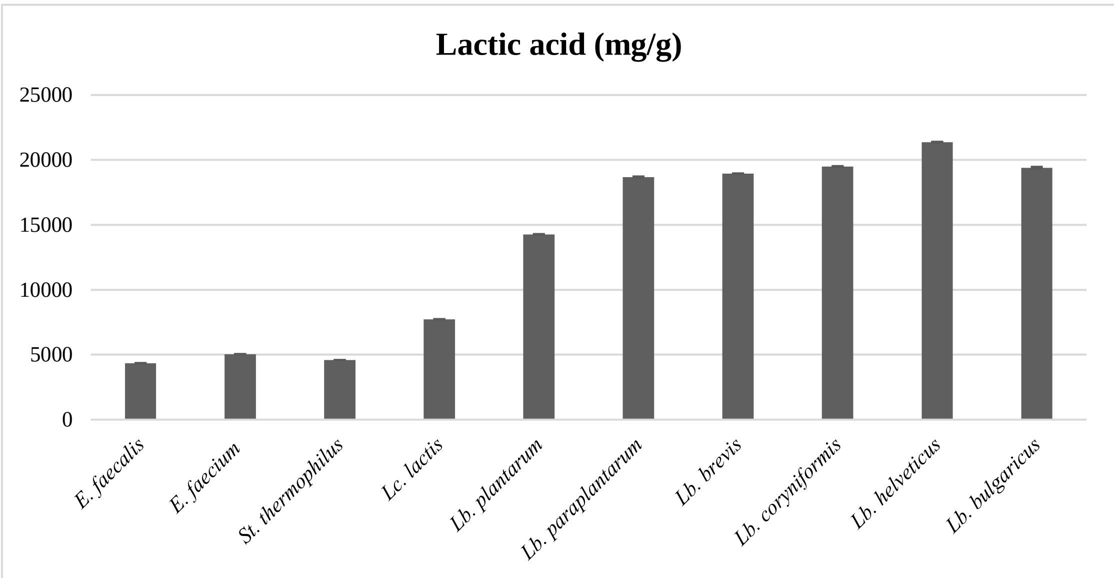

Figure 4.4 Lactic acid concentration of each postbiotic

According to the observed HPLC results, all Lactobacillus species produced comparatively large amounts of lactic acid. *Lb. helveticus* 9-B_5 produced the highest amount of 21368.32 mg/g followed by that of *Lb. coryniformis* and *Lb. bulgaricus*. The results observed for the postbiotics of *Lb. helveticus* 9-B_5 were contrary to the findings of Rozhkova et al. (2023), who couldn't observe any lactic acid production by *Lb. helveticus* 9-B_5. This could be due to the difference in the characteristics of various strains of the same bacteria. The observed quantities of lactic acid produced by all LAB were deemed to be statistically similar. Since the production of lactic acid is a characteristics of LAB hence other studies including that from Mani-López et al. (2022) also confirmed the production of lactic acid by LAB species.

In another study, the results obtained for the postbiotics of *Lb. helveticus* 9-B_5 are in line with the findings of Jamaran et al. (2021), for *Lb. reuteri* postbiotics (same genera) where the postbiotics obtained from *Lb. reuteri* were highest among others.

The production of lactic acid by *Lb. plantarum* was much lower than that of other Lactobacillus species. These findings are different from that described by (Sheeladevi & Ramanathan, 2011). Difference in incubation time and glucose quantity could be a contributing factor for the decreased production of lactis acid. Also, high production of lactic acid by *Lb. plantarum* is achieved at 37 ºC temperature, pH 5.5, 10% inoculum level and 72 hours of incubation time (Sheeladevi & Ramanathan, 2011). None of the above conditions were similar to the conditions provided in our study hence, comparatively lower amounts of lactic acid was achieved.

LAB species like *St. thermophilus*, *Lc. lactis* and Enterococcus species produced lower amounts of lactic acid. This might be because of further metabolism of lactic acid due to the formation of cytochromes by hemes as stated by (König & Fröhlich, 2017). Although lactic acid was produced by *Lc. lactis*, but its amount was much lower as compared to other. This could be due to the fact that since the moment when lactic acid is produced by LAB strain, the pH of the environment is reduced which slows the metabolism of bacterial cells to produce more metabolites including lactic acid (Singhvi, Zendo, & Sonomoto, 2018).

4.2.4.5 Formic acid

Formic acid is one of the simplest carboxylic acid having only a single carbon atom and is an important antibacterial agent. Limited quantities of formic acids are found to be present in UHT and pasteurized milks that might increase over time (Edwards, Badiger, Heldman, & Klein, 2021).

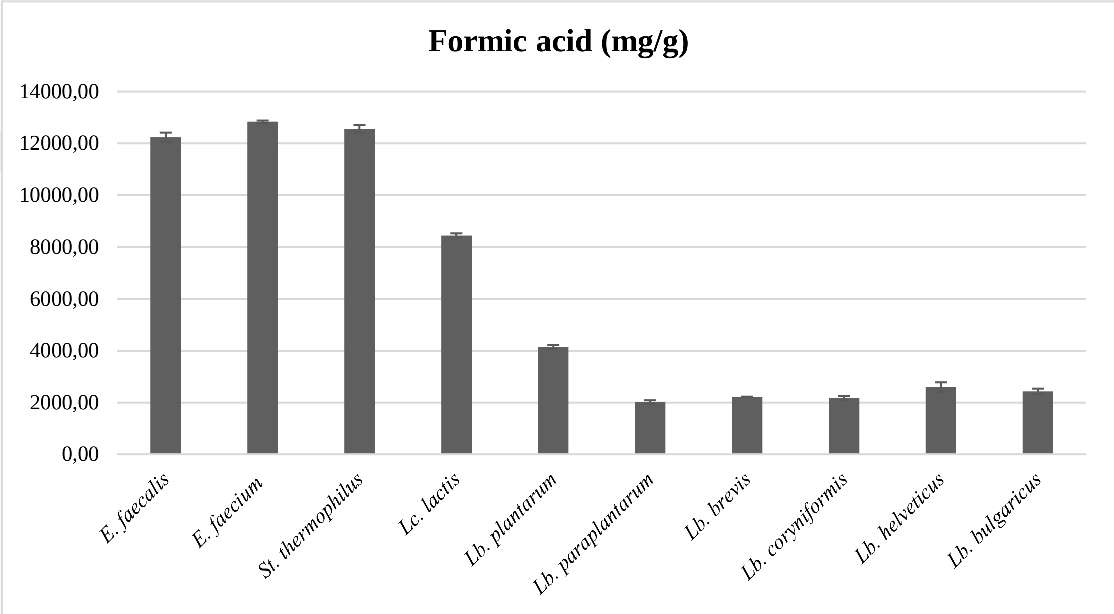

Figure 4.5 Formic acid concentration of each postbiotic

According to the obtained results, Enterococcus species i.e., *E. faecalis* and *E. faecium* produced the highest amounts of formic acid as 12232.20 mg/g and 12835.11 mg/g, respectively. Significantly larger amounts of 12561.75 mg/g was also observed for *St. thermophilus 1-K*$_4$. Meanwhile, postbiotics produced by Lactobacillus species had much lower concentrations of formic acid. The presence of formic acid (due to initial production by Lactobacillus) in the growth environment causes the retardation of LAB proliferation hence, inhibit them to produce more formic acid (Plumed-Ferrer & von Wright, 2011).

Formic acid is produced in high amounts from pyruvate via pyruvate formate-lyase pathway, in substrates having galactose instead of glucose. Since, MRS broth used for the incubation of Lactobacillus consists of glucose as the main sugar instead of galactose so it is thought that the activity of PFL enzyme was carried out inefficiently (Melchiorsen, Jokumsen, Villadsen, Israelsen, & Arnau, 2002).

Contrary to this, some LAB species like *Lb. sanfrancisco* CB1 also produce considerable amounts of formic acid by pyruvate via pyruvate formate-lyase system (Corsetti, Gobbetti, Rossi, & Damiani, 1998). According to Noureldein et al. (2020), it was also observed that the amounts of formic acids produced by *Lb. brevis* were not high as compared to other acids produced by the same bacteria. This could be due to the conversion of formic acid into other compounds.

4.2.4.6 Acetic acid

Acetic acid is a volatile acid which is commonly known as vinegar acid. It has similar characteristics of being antimicrobial and antifungal agent, as that of other organic acids but has limited utilization in milk processing. One of the main reason is it coagulates the milk casein proteins and cause aggregation of peptides. But due to the same reason it has potential to be used in the manufacturing of other dairy products like cheese (Tirloni et al., 2020).

The HPLC results for all postbiotics showed almost similar amounts of acetic acid that were detected at 210 nm which shows that most LAB species including Enterococcus, Lactococcus and Lactobacillus produce some quantity of acetic acid during incubation. This amount of acetic acid might vary with the type of bacterial strain as well as the growth medium used.

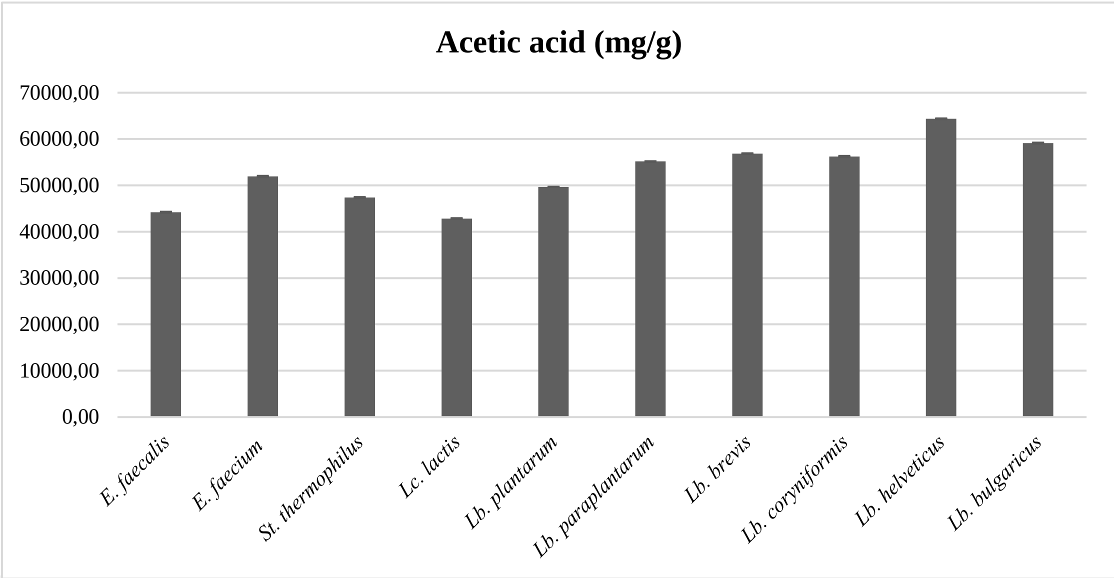

Figure 4.6 Acetic acid concentration of each postbiotic

Although all experimented LAB species were able to produce high amount of acetic acid but when compared to the findings of Özcelik et al. (2016), the detected amount of acetic acid was near to zero in *Lb. plantarum*, *Lc. lactis* and *St. thermophilus* that might occur due to the use of different bacterial strain. Much lower amounts of acetic acid was produced by *St. thermophilus* and *Lc. lactis* strains extracted from cheese made from sheep milk could be possible due to the difference in source from which LAB was obtained (Hladíková et al., 2012).

The production of acetic acid and its presence in postbiotic of *E. faecalis* was also confirmed by Hussain et al. (2021), but its concentration was much higher as compared to our data which could be due to the different strains which might produce higher organic acid amounts as compared to others.

The highest amount was detected in postbiotics from *Lb. helveticus* 9-B_5 followed by those produced by *Lb. bulgaricus*. The results for postbiotics of *Lb. helveticus* 9-B_5 are much similar to the results obtained by Rozhkova et al. (2023), who also showed higher acetic acid production in growth medium of MRS broth. The production of acetic acid in MRS could be higher due to the presence of glucose used as an energy source by bacteria.

Adequate amount of acetic acid produced by *Lb. plantarum* could be attributed due to the activation of phosphogluconate pathway and the intra-route of acetate kinase enzyme which could contribute to the production of acetic acid, as also briefly explained by (Filannino et al., 2014).

4.2.4.7 Propionic acid

Propionic acid is a short chain fatty acid that is usually found in foods as a food additive and food preservative. It is mostly used in Emmental and other semi-hard Swiss type cheeses where it provides both antimicrobial effect (reported against *L. monocytogenes*) and preferred sensory properties (nutty flavor and harder texture) to the cheese (Wemmenhove, van Valenberg, Zwietering, van Hooijdonk, & Wells-Bennik, 2016).

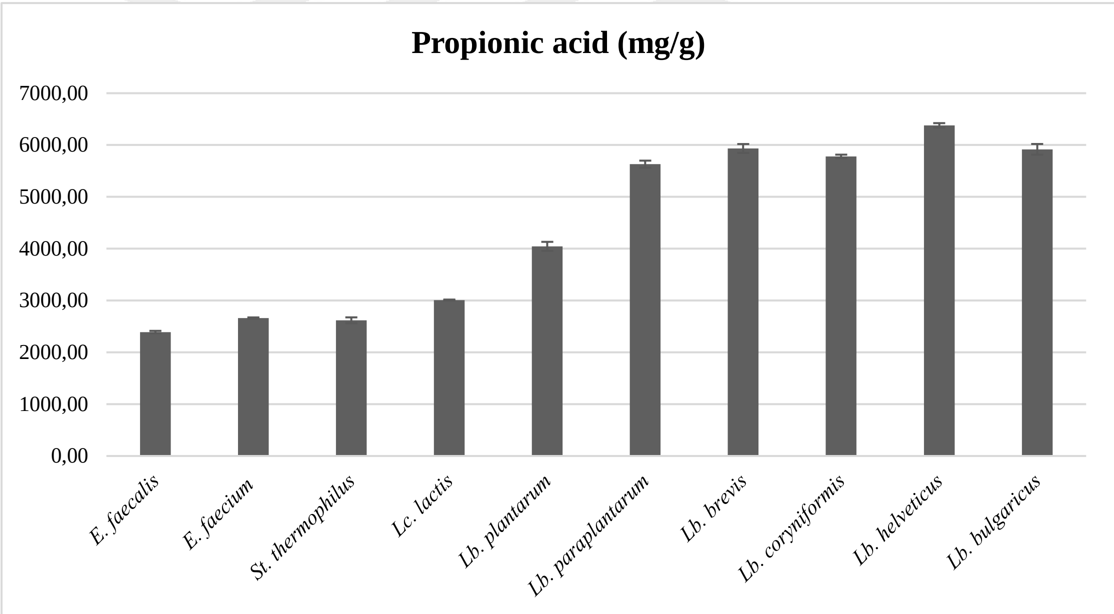

Figure 4.7 Propionic acid concentration of each postbiotic

Although propionic acid production is the characteristic of propionic acid bacteria, some other LAB can also produce propionic acid during incubation. The production of propionic acid was observed by adding mixture of *Lb. rhamnosus* and *Propionibacterium freudenreichii* to yogurts, where LAB species were also deemed to be a contributing factor in producing some propionic acid (Suomalainen & Mäyrä-Makinen, 1999).

Mainly Lactobacillus species were observed to produce higher amounts of propionic acid compared to Enterococcus, Lactococcus and Streptococcus strains. Best propionic acid producer was determined as *Lb. helveticus* 9-B_5 (6379.06 mg/g) among the Lactobacillus strains. This high production of propionic acid by *Lb. helveticus* 9-B_5 is similar to the findings of Behbahani et al. (2023) for *Lb. fermentum* which confirms the production of this organic acid by lactobacillus species.

Propionic acid was produced in lower concentrations by both enterococcus strains. Propionic acid could also be produced as a byproduct by the reaction of certain amino acids (Urdaneta et al., 1995). Since incubation was done in M17 broth without the presence of any amino acids, the production of propionic acid was limited.

4.2.4.8 Butyric acid

Butyric acid is a common short chain fatty acid that was initially detected from rancid butter. It has wide utilization as food preservative and additive with additional anticancer properties stated by (Nudelman et al., 1992).

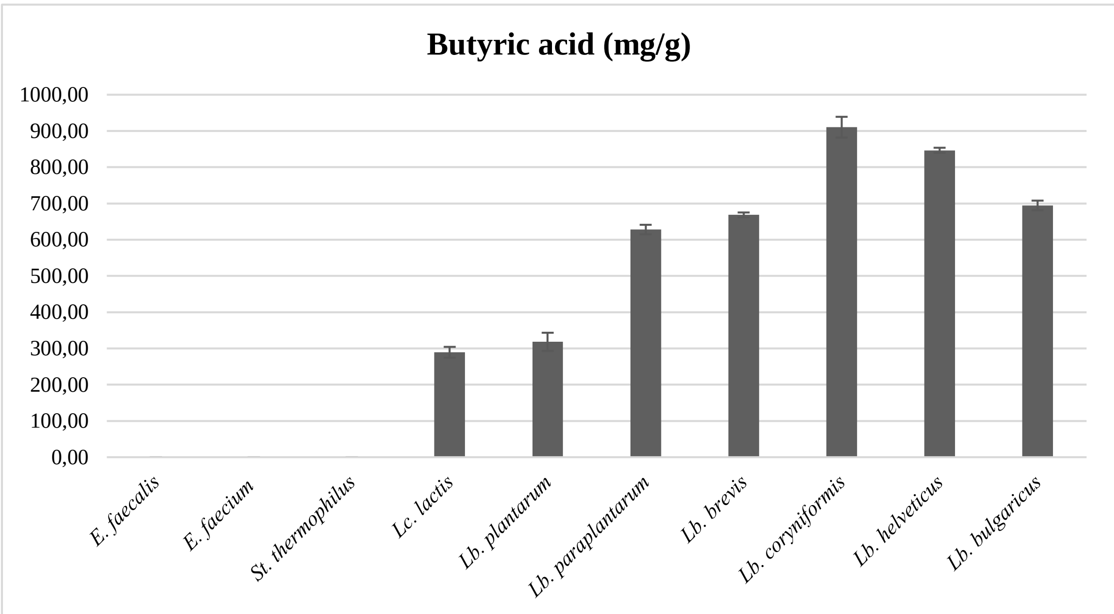

Figure 4.8 Butyric acid concentration of each postbiotic

Butyric acid was not detected in postbiotics both from Enterococcus and Streptococcus species. However, all the Lactobacillus and Lactococcus species were found as butyric acid producer, although in low concentrations. Among all LAB highest quantities were determined in the postbiotics of *Lb. coryniformis* (910.41 mg/g) and *Lb. helveticus* 9-B_5 (846.54 mg/g), respectively. Some amount of butyric acid was also detected in the postbiotic of *Lb. plantarum*. There is an important link between the production of butyric acid with the presence of yeast extract in MRS broth which positively contribute to the availability of biomass for bacterial strains (Aiello et al., 2023).

The production of butyric acid by LAB is not linked to the presence of glucose as the sugar source (Nazzaro, Fratianni, Nicolaus, Poli, & Orlando, 2012). Also, Nazzaro et al. (2012) indicates the positive effect of butyric acid in providing carbon source to other LAB to encourage them to produce butyric acid. The production of butyric acid by *Lb. plantarum* and *Lb. brevis* was also observed by Kam et al. (2011) and Sakai et al. (2007), but these were in small amounts which showed that the MRS medium without enrichment is not sufficient.

4.2.4.9 Acetoin

Acetoin is an aromatic compound mostly added to the food to enhance flavor and aroma, especially responsible for the aroma in yogurt (Tian, Yu, Yu, & Chen, 2020). Acetoin is mostly produced via citrate cycle in the presence of citrate and is present during cheese ripening to impart flavor (La, 2014). Acetoin is also produced by many homofermentative LAB species where these bacteria convert pyruvate to acetoin with the production of carbon dioxide as described by Walker (1959).

According to the obtained results, acetoin was produced by all Lactobacillus species at some quantities while postbiotics of Enterococcus species, *St. thermophilus* 1-K_4 and *Lc. lactis* showed minimum amounts of acetoin. Highest acetoin amount was produced by *Lb. paraplantarum* (20722.50 mg/g) while the lowest amount was detected in postbiotics of *St. thermophilus* 1-K_4 (1282.30 mg/g). The larger production of acetoin occurs via the pyruvate metabolism in lactobacillus bacteria like *Lb. arabinosus* and also from *Lb. brevis* (Walker, 1959).

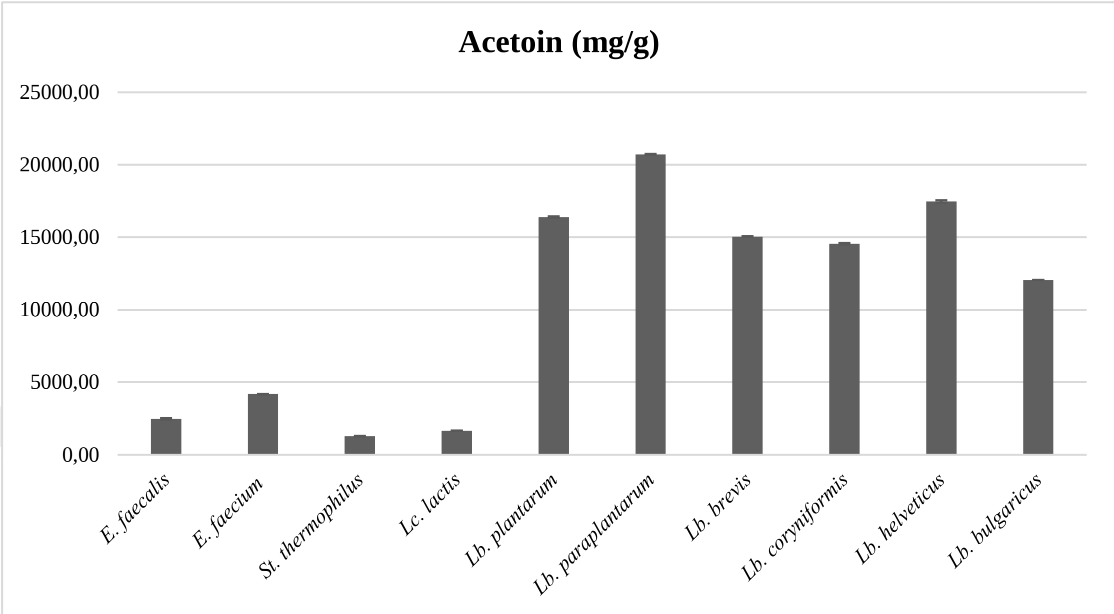

Figure 4.9 Acetoin concentration of each postbiotic

The lowest amount of acetoin was detected in postbiotics of *St. thermophilus* which might be explained due to the absence or suppression of certain enzymes like ɑ-acetolactate synthase and decarboxylase (de Souza Oliveira, Torres, Perego, de Oliveira, & Converti, 2012). Followed by postbiotics of *St. thermophilus*, postbiotics from *Lc. lactis* also showed minimum amounts of acetoin. The probable reason for this is the enzymatic conversion of acetoin of diacetyl compound as stated by Gänzle & Gobbetti (2012).

Additionally, the process of conversion of acetoin to diacetyl by some streptococcus species (*St. lactis*) via utilization of $NADH_2$ could also contribute to the decreased amount of *St. thermophilus* in its cell-free extract/ postbiotics (Chuang & Collins, 1968).

The presence of acetoin has also been confirmed by the findings of Chang et al. (2021) who showed that all the studied strain of *Lactiplantibacillus plantarum* produced some significant amounts of acetoin in MRS broth formulated with varying concentrations of yeast extract, magnesium sulfate tetrahydrate and other components in in-vitro environment. While considering the production of acetoin by *Lb. brevis*, it was observed that *Lb. brevis* utilized pyruvate to produce acetoin (Chuang & Collins, 1968). Comparing with figure 4.2 it was deemed that high amount of pyruvic acid could be a contributing factor for the adequate production of acetoin by *Lb. brevis*.

4.2.4.10 Diacetyl

Diacetyl, also known as 2,3 butanediol, is another flavor enhancer produced by many LAB species specific to dairy products. It imparts a sweet, creamy, and buttery flavor to food commodity, hence increases its sensory properties.

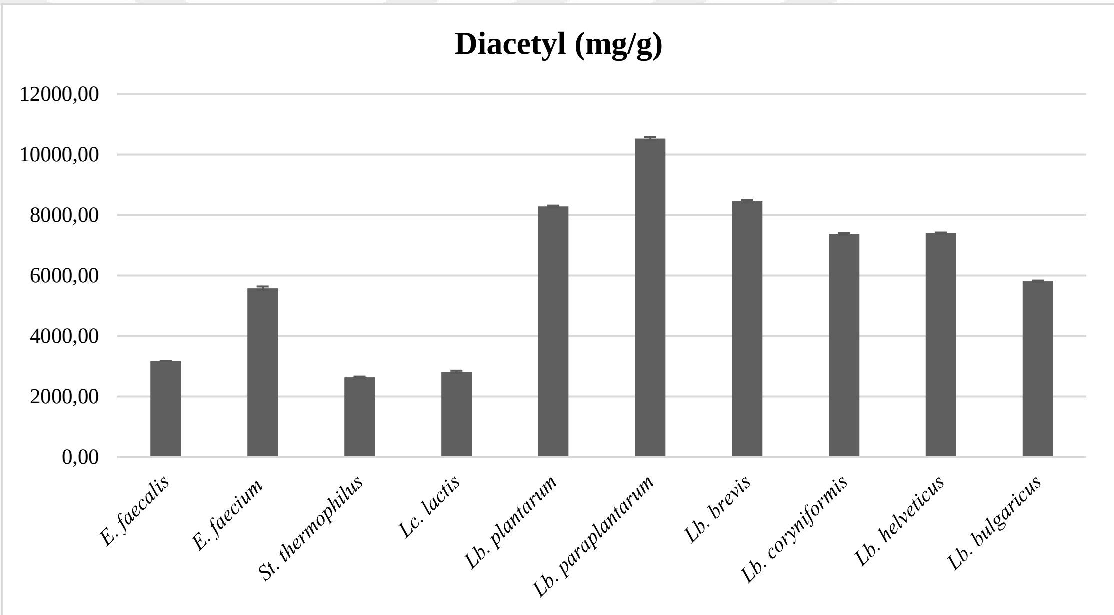

Figure 4.10 Diacetyl concentration produced by each postbiotic

The enzymes present in the LAB are responsible for the production of diacetyl in the presence of pyruvate and citrate (Magee Jr, Olson, & Lindsay, 1981). The production of diacetyl is closely linked to the presence of pyruvate in the cell of microorganism. Hence, the amount of diacetyl is deemed to be linked with the presence of pyruvic acid (Speckman & Collins, 1968). According to the obtained data, diacetyl was detected in a higher amount in the postbiotics of *Lb. paraplantarum* while the lowest quantity was obtained in the postbiotics of *St. thermophilus* 1-K_4.

Small amounts of diacetyl produced by *St. thermophilus* was contributed by the presence of pyruvate and its deemed conversion to acetoin and diacetyl as shown by (Chammas, Saliba, Corrieu, & Béal, 2006).

Additionally, reasonably higher amount of diacetyl was detected in the postbiotics of Lactobacillus species as compared to Enterococcus species. Although pyruvate is the main source of diacetyl but much smaller quantities of acetoin and diacetyl are also obtained from *Lb. plantarum* and *Lb. brevis* in the presence of citrate in succinic buffer instead of pyruvate (El-Gendy, Abdel-Galil, Shahin, & Hegazi, 1983).

Diacetyl is produced when excess pyruvate is present in the environment that can be converted to α-acetolactate (Gänzle & Gobbetti, 2012). LAB species like *Lc. lactis* reduces this α-acetolactate to acetoin when is again converted to diacetyl. Since, it is observed from figure 4.2 that minimum amount of pyruvic acid is present in the postbiotics of *Lc. lactis*, it also indicates lower pyruvate hence, we could describe this as the probable cause of lower diacetyl compounds.

Diacetyl is produced in acidic conditions where is utilizes pyruvate and is synthesized into diacetyl during transcription phase as discussed by (Filannino et al., 2014). García-Quintáns et al. (2008) also reported the same mechanism of production of diacetyl from pyruvate via citrate transport system by *Lc. lactis subsp. lactis* (bv. diacetylactis CRL264). This was possible because of the presence of genes which are expressed only in acidic situations.

High amount of diacetyl was observed from the postbiotics of *Lb. paraplantarum* followed by the postbiotics of *Lb. plantarum*. This intense production of diacetyl by *Lb. plantarum* was also evident from the findings of Ricci et al. (2018) who observed that *Lb. plantarum* (strain 1486) extracted from various pecorino cheese was able to produce adequate amounts of diacetyl from citrate.

Table 4.5 The amounts of organic acids in each postbiotics

Sample	*Lb. bulgaricus*	*Lb. helveticus* 9-B_5	*Lb. coryniformis*	*Lb. brevis*	*Lb. paraplantarum*	*Lb. plantarum*	*Lc. lactis*	*St. thermophilus* 1-K	*E. faecium*	*E. faecalis*
Citric acid	992.8 ± 3.7^{d}	1061.3 ± 1.3^{c}	1183.9 ± 0.9^{b}	1482.3 ± 16.9^{a}	1209.4 ± 11.9^{b}	882.4 ± 44.6^{e}	454.0 ± 4.6^{f}	347.4 ± 9.8^{g}	406.7 ± 9.1^{fg}	272.7 ± 3.2^{h}
Pyruvic acid	991.3 ± 18.4^{c}	1531.2 ± 41.7^{b}	1317.7 ± 33.9^{b}	2363.5 ± 194.0^{a}	1581.7 ± 47.6^{b}	1362.3 ± 22.2^{b}	531.0 ± 42.9^{d}	167.7 ± 17.9^{e}	309.0 ± 71.2^{de}	199.0 ± 19.8^{e}
Succinic acid	317.3 ± 14.0^{f}	277.3 ± 38.1^{fg}	100.4 ± 2.1^{h}	177.8 ± 20.2^{fgh}	152.7 ± 24.7^{gh}	1850.1 ± 20.8^{e}	2440.5 ± 89.8^{d}	3766.3 ± 6.7^{c}	5808.6 ± 29.3^{a}	5249.4 ± 42.0^{b}
Lactic acid	19400.3 ± 73.3^{b}	21368.3 ± 33.7^{a}	19489.1 ± 41.6^{b}	18945.3 ± 22.7^{c}	18669.0 ± 62.1^{d}	14268.1 ± 28.5^{e}	7734.4 ± 14.5^{f}	4585.1 ± 7.4^{h}	5045.6 ± 3.2^{g}	4350.8 ± 1.2^{i}
Formic acid	2425.5 ± 109.9^{ef}	2586.5 ± 190.4^{e}	2166.7 ± 75.7^{ef}	2220.9 ± 6.6^{ef}	2015.5 ± 68.5^{f}	4132.9 ± 79.9^{d}	8444.8 ± 81.7^{c}	12561.8 ± 140.5^{ab}	12835.1 ± 45.7^{a}	12232.2 ± 183.5^{b}
Acetic acid	59127.7 ± 100.3^{b}	64369.4 ± 86.4^{a}	56230.2 ± 132.1^{d}	56853.0 ± 82.0^{c}	55159.9 ± 61.6^{e}	49680.9 ± 25.5^{g}	42854.4 ± 73.3^{j}	47356.0 ± 125.0^{h}	51913 ± 148.7^{f}	44201.9 ± 111.6^{i}
Propionic acid	5916.6 ± 103.4^{b}	6379.1 ± 43.4^{a}	5779.4 ± 35.0^{bc}	5933.0 ± 86.0^{b}	5630.5 ± 68.5^{c}	4044.7 ± 87.0^{d}	3009.5 ± 8.9^{e}	2618.9 ± 55.9^{fg}	2659.7 ± 14.0^{f}	2390.6 ± 23.7^{g}
Butyric acid	694.5 ± 13.3^{c}	846.5 ± 7.2^{b}	910.4 ± 28.7^{a}	668.4 ± 6.7^{cd}	628.1 ± 13.1^{d}	318.2 ± 25.1^{e}	289.5 ± 14.9^{e}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}
Acetoin	12055.8 ± 23.3^{f}	17481.3 ± 82.8^{b}	14563.4 ± 63.5^{e}	15055.5 ± 42.7^{d}	20722.5 ± 40.7^{a}	16396.4 ± 49.5^{c}	1665.3 ± 17.9^{i}	1282.3 ± 35.2^{j}	4184.1 ± 24.9^{g}	2472.6 ± 58.9^{h}
Diacetyl	5806.6 ± 24.3^{e}	7403.0 ± 16.8^{d}	7372.9 ± 23.3^{d}	8451.8 ± 36.4^{b}	10526.4 ± 49.0^{a}	8280.9 ± 30.1^{c}	2810.2 ± 39.7^{h}	2632.3 ± 22.9^{i}	5573.3 ± 61.9^{f}	3168.3 ± 3.7^{g}

The differences ($P < 0.05$) among all samples were indicated with lower-case letters (a, b, c, ab etc.). Values are shown as Mean ± SD.

4.3 Determination of Bioprotective Role of Postbiotics in Pasteurized Milk During Storage

4.3.1 pH values

The pH of pasteurized milk ranges between 6.6 to 6.8. This pH is neither too acidic nor basic but lies near to neutrality. Due to the presence of organic acid formations might slightly decrease the pH value of milk. The following table shows the pH values of pasteurized milk on day 1, 5 and 10 which treated with each postbiotic obtained from LAB species.

Table 4.6 pH values of pasteurized milks during the storage period

Sample	**Storage Day**		
	1	**5**	**10**
Control	6.82 ± 0.00^{a}	6.77 ± 0.00^{abcd}	6.64 ± 0.00^{gh}
E. faecalis	6.76 ± 0.03^{abcde}	6.78 ± 0.01^{abcd}	6.73 ± 0.01^{cdef}
E. faecium	6.79 ± 0.01^{abc}	6.73 ± 0.01^{cdef}	6.34 ± 0.01^{i}
***St. thermophilus* 1-K$_4$**	6.76 ± 0.03^{abcde}	6.82 ± 0.02^{ab}	6.66 ± 0.05^{fgh}
Lc. lactis	6.75 ± 0.01^{abcde}	6.77 ± 0.00^{abcd}	6.32 ± 0.02^{i}
Lb. plantarum	6.75 ± 0.02^{abcde}	6.80 ± 0.02^{abc}	6.71 ± 0.02^{defg}
Lb. paraplantarum	6.75 ± 0.01^{abcde}	6.79 ± 0.02^{abc}	6.69 ± 0.01^{efgh}
Lb. brevis	6.75 ± 0.02^{abcde}	6.79 ± 0.01^{abc}	6.61 ± 0.01^{h}
Lb. coryniformis	6.75 ± 0.01^{abcde}	6.80 ± 0.03^{abc}	6.76 ± 0.03^{abcde}
***Lb. helveticus* 9-B$_5$**	6.75 ± 0.02^{abcde}	6.79 ± 0.01^{abc}	6.62 ± 0.02^{h}
Lb. bulgaricus	6.74 ± 0.01^{bcde}	6.78 ± 0.01^{abcd}	6.70 ± 0.00^{defg}

An interaction was found between the storage time and samples ($P < 0.05$). This interaction was indicated with lower-case letters (a, b, ab etc.). Values are shown as Mean ± SD.

According to the obtained data, at the end of storage period all the pH values were similar except for samples in which the postbiotics of *Lc. lactis* and *E. faecalis* were added. Significant relationship was observed between the storage time and samples (P<0.05). Sample containing postbiotics of *E. faecalis* showed decrease in pH during storage. This lowering of pH is a general trend which is deemed to be shown by many *E. faecalis* strains including *E. faecium* OV3 and bacteriocins of this strain might play a major role in changing the pH values during storage (Choeisoongnern et al., 2023). Also, it is seen that the antimicrobial activity of cell-free supernatant of *E. faecium* strains remain intact at lower pH values for *E. faecium* ST88Ch (Pingitore, Todorov, Sesma, & de Melo Franco, 2012). A similar pH decrease is also observed for *E. faecium* where pH was decreased to 6.34 at 10th day of storage. The antimicrobial activity of LAB remain intact at lower pH (between 4-6) while it could decrease up to 50% as the pH increases (Moradi et al., 2019).

Comparisons made at day 1, 5 and 10 showed no significant changes between pH of *Lb. bulgaricus*, *St. thermophilus* 1-K_4, *Lb. plantarum* and *Lb. paraplantarum*. The results for postbiotics of *Lb. plantarum* were also similar to the finding of Jo et al. (2021), who tested the meat product naturally preserved with postbiotics of *Lb. plantarum* (SKD4). The observations were similar in terms of initial increase and later decrease in pH during storage period. The addition of cell-free supernatant from *Lb. plantarum* Cys5-4 didn't cause any change in the pH value of the beverages (Tenea & Barrigas, 2018).

Also, many studies reveal that the pH range between 3-6 is essential in maintaining the antimicrobial activity of cell-dress extracts. The effectiveness of postbiotics is considerably lost at the alkaline pH level of 9 (Qadi et al., 2023). Hence, it is deemed that decrease in pH of samples containing *St. thermophilus* and *Lc. lactis* is linked due to the presence of antimicrobial compounds (organic acids) in their postbiotics.

Additionally, the loss of antimicrobial activity of cell-free supernatant of *Lc. lactis* could be observed even at a slight increase of pH to 6.5 (Iranmanesh, Ezzatpanah, & Mojgani, 2014). This loss of activity could be due to the loss of effectiveness of organic acids. On day 10, pH of the sample with addition of postbiotics from *Lc. lactis* was significantly decreased to 6.32, the occurrence that is commonly associated with *Lc. lactis* (Frees, Vogensen, & Ingmer, 2003). The reason for this increase in acidity and lowering of pH is due to the production of high amounts of organic acids like lactic acid (Behbahani, Jooyandeh, Vasiee, & Zeraatpisheh, 2023).

4.3.2 Titratable acidity (LA %)

Lactic acid % of pasteurized milk ranges between 0.014 to 0.016. The following table shows the effect of addition of postbiotics on LA % of pasteurized milk during 10-days of storage period. The values were observed to compare the relationship between each sample, and a significantly higher difference was seen between all samples. The storage time for all these postbiotics also showed relatively higher significantly different values. LA % is directly affected by many factors including reaction of caseins, acid phosphates, organic acids causing lipolysis of fat globules and indirectly effected by feeding, type of feed, stress factors etc. (Calamari, Gobbi, & Bani, 2016).

Table 4.7 Titratable acidity (LA %) values of pasteurized milks during the storage period

Sample	**Storage Time**		
	1	**5**	**10**
Control	0.014 ± 0.00^{bB}	0.013 ± 0.00^{bB}	0.014 ± 0.00^{bA}
E. faecalis	0.014 ± 0.001^{abB}	0.014 ± 0.001^{abB}	0.015 ± 0.001^{abA}
E. faecium	0.014 ± 0.00^{aB}	0.015 ± 0.002^{aB}	0.017 ± 0.001^{aA}
***St. thermophilus* 1-K_4**	0.014 ± 0.001^{abB}	0.014 ± 0.00^{abB}	0.014 ± 0.00^{abA}
Lc. lactis	0.013 ± 0.00^{abB}	0.015 ± 0.001^{abB}	0.016 ± 0.00^{abA}
Lb. plantarum	0.014 ± 0.001^{bB}	0.013 ± 0.00^{bB}	0.014 ± 0.00^{bA}
Lb. paraplantarum	0.014 ± 0.00^{abB}	0.013 ± 0.00^{abB}	0.015 ± 0.001^{abA}
Lb. brevis	0.014 ± 0.001^{abB}	0.014 ± 0.00^{abB}	0.015 ± 0.001^{abA}
Lb. coryniformis	0.014 ± 0.001^{bB}	0.013 ± 0.00^{bB}	0.014 ± 0.00^{bA}
***Lb. helveticus* 9-B_5**	0.013 ± 0.00^{abB}	0.014 ± 0.001^{abB}	0.016 ± 0.001^{abA}
Lb. bulgaricus	0.013 ± 0.00^{abB}	0.014 ± 0.00^{abB}	0.016 ± 0.001^{abA}

The differences ($P < 0.05$) among all samples were indicated with lower-case letters (a, b, ab etc.) while the differences between storage time were indicated with upper-case letters (A, B). Values are shown as Mean ± SD.

According to the extracted data, LA % of samples containing postbiotics from *St. thermophilus* 1-K_4, *Lb. plantarum*, and *Lb. coryniformis* remained same and showed no deviation on day 1 while a significant increase ($P < 0.05$) was detected for samples having postbiotics of *E. faecium* and *Lc. lactis*. As observed in Table 4.6, samples having postbiotics of *E. faecium* showed lowest pH values during 10th day of storage (6.34 ± 0.01) which might be because of higher LA % as compared to other samples.

A gradual increase in the titratable acidity of pasteurized milk by adding LAB metabolites has also been observed in other studies. The observed increase might occur due to the presence of heat resistant lactic acid bacteria which continue to perform activity during storage (E. M. Ibrahim & Elbarbary, 2012). No significant differences ($P > 0.05$) were observed between day 1 and day 5, however significant difference ($P < 0.05$) was detected at day 10. Minimum change in total acidity of pasteurized milk is observed by the addition of bacterial metabolites (Wirjantoro & Lewis, 1996).

A slight increase in TA % was observed for sample containing postbiotics of *Lb. helveticus* 9-B_5. According to Ibrahim et al. (2023), this change is contributed by the constituents of milk like amino acid profile and fatty acids – the lates being responsible for causing hydrolysis of fats in milk. The increase in acidity is also observed in cheese samples made with 1% of CFS of *Lb. helveticus* (Atia, Aly, Metwally, Hassanein, & Elshishtawy, 2022).

In samples containing postbiotics of *St. thermophilus*, no change was observed in TA % during storage. Inconsiderable change in TA % was observed which means that yogurts having postbiotics from *St. thermophilus* are not affected during 15 day storage (Sadighbathi, Amiri, Saris, & Yousefvand, 2023). Slight increase in TA % is seen in yogurts having postbiotics of *Lb. bulgaricus* (Sadighbathi et al., 2023).

4.3.3 Total solid (%)

The total solid in pasteurized milk is in the form of proteins, fats, carbohydrates, and minerals, etc. The percentage of total solid reflects the quality of milk. The below table shows the difference in total solid % during storage.

Table 4.8 Total solid content (%) of pasteurized milks during the storage period

Sample	Storage Day		
	1	5	10
Control (PS)	11.35 ± 0.00^{bcdefgh}	11.70 ± 0.00^{abcdefgh}	11.49 ± 0.17^{bcdefgh}
E. faecalis	10.53 ± 0.13^{defgh}	12.55^{abc} ± 0.20^{abc}	10.23 ± 1.34^{gh}
E. faecium	9.97 ± 0.07^{h}	13.45 ± 0.34^{a}	11.37 ± 0.41^{bcdefgh}
***St. thermophilus* 1-K$_4$**	10.51 ± 0.26^{defgh}	12.79 ± 0.11^{ab}	11.23 ± 0.38^{bcdefgh}
Lc. lactis	10.49 ± 0.56^{defgh}	12.38 ± 0.11^{abc}	11.28 ± 0.56^{bcdefgh}
Lb. plantarum	10.44 ± 0.38^{efgh}	12.43 ± 0.13^{abc}	10.51 ± 0.93^{defgh}
Lb. paraplantarum	10.34 ± 0.04^{gh}	12.35 ± 0.27^{abc}	11.26 ± 0.18^{bcdefgh}
Lb. brevis	10.48 ± 0.05^{defgh}	12.36 ± 0.63^{abc}	11.30 ± 0.53^{bcdefgh}
Lb. coryniformis	10.40 ± 0.26^{fgh}	12.25 ± 0.12^{abcd}	11.27 ± 0.60^{bcdefgh}
***Lb. helveticus* 9-B$_5$**	10.36 ± 0.33^{gh}	12.20 ± 0.60^{abcdef}	11.80 ± 0.29^{abcdefg}
Lb. bulgaricus	10.85 ± 0.17^{cdefgh}	12.22 ± 0.16^{abcde}	10.82 ± 0.57^{cdefgh}

A significant interaction was found between storage day and samples (P < 0.05) which was indicated with lower-case letters (a, b, ab etc.). Values are shown as Mean ± SD. PS = pasteurized milk.

Significant differences were observed both among samples and among storage days (P<0.05). The control remained unsignificant whose results are also similar to the results described by Correa et al. (2019) while sample with *Lb. bulgaricus* postbiotics also showed similar behavior during a 10-day storage time.

Correa et al. (2019) found the percentage of TS to be around 11.39 % - 11.49 % in pasteurized milk, while in some other literatures it varies between 8.41 % to 9.34 % (Zebib, Abate, & Woldegiorgis, 2023). The results of Correa et al. (2019) were similar to our findings for control sample and the other samples containing postbiotics of Lactobacillus species. The TS % of *Lb. bulgaricus* was similar at day 1 and day 10 of storage. Additionally, all samples showed a significant increase on day 5 of storage with the increase being the highest for samples having *E. faecium* postbiotics. The statistically significant difference is also evident as they share no uppercase letter. While total solid

% of all samples showed slightly similar behavior at day 1 and day 10, but a highly significant difference was observed at day 5.

The decrease in TS % is generally linked with the utilization of carbohydrate source i.e., lactose to lactic acid (Degrain et al., 2020). This is mostly done during fermentation process but since, only postbiotic metabolites are involved in this study it could be deemed that enzymes secreted by these bacteria played a major role in carrying out bioconversion of lactose to lactic acid and hence causes decrease in TS %.

The slight increase in the TS % of all samples having the postbiotics might arise from the presence of exopolysaccharides in the postbiotics of these native LAB. These exopolysaccharides bind to some milk constituents and cause some increase in TS content. The addition of these exopolysaccharides enhances the water holding capacity (WHC) of dairy products which is later highlighted in the TS % (Patel & Prajapati, 2013).

4.3.4 Rheological factors

The rheological factors of all the samples were calculated using Power law model. Consistency Index (K), and Flow behavior index (n) were observed on day 1, 5, and 10 during storage time. These results are described in the Table 4.9;

According to Bienvenue et al. (2003), milk shows Newtonian behavior under normal conditions. In pasteurized milks high temperature often causes some degradation of protein hence increasing the viscosity (Ketto, Johansen, Skeie, & Schüller, 2018). Even if the TS % is low, initial high temperatures cause a shift of behavior from newtonian to non-newtonian (Trinh, Trinh, & Haisman, 2007). The denaturation of β-lactoglobulins occur during HTST pasteurization of milk. These denatured β-lactoglobulins interact with casein micelles and other denatured whey proteins hence, causing the change in viscosity during storage (Li, Joyner, Lee, & Drake, 2018).

No significant differences were obtained among samples in terms of K and n values ($P > 0.05$). However, significant differences were found in the same values during storage period ($P < 0.05$). The consistency index (K) is often calculated in Pa.s. Samples with postbiotic of *E. faecalis* showed highest K values of 0.640 Pa.s on day 1 which decreased to 0.185 Pa.s on the 10th day. For samples having postbiotics from *E. faecium* and *Lb. bulgaricus* the value of consistency index (K) sharply decreased at day 10 of storage. As indicated in Table 4.7 the acidity also increased at 10^{th} day of storage causing the textural changes in sample having postbiotics of *Lb. bulgaricus*. This sharp decline in consistency index (K) was also observed in milk fermented with *Lb. bulgaricus* and could be explained by the increase in acidity (TA %) during storage period (Karsheva, Paskov, Tropcheva, Georgieva, & Danova, 2013).

Some samples that have postbiotics of *Lb. coryniformis*, *Lb. paraplantarum*, and *Lb. plantarum*, the value of K showed same decline pattern during storage which could be explained by the increase in TA % during storage. For samples having postbiotics of *Lc. lactis*, the decrease in consistency index was minimum due to the low production of various organic acids as compared to other LAB. In samples having postbiotics of *Lb. helveticus* 9-B_5, the decrease in consistency index at 10^{th} day of storage could be linked with the presence of large amounts of organic acids which could cause textural changes in the sample. Similarly, in sample having postbiotics of *Lb. brevis*, the higher total organic acid content and high percentage of acetic acid and lactic acid resulted in significant decline of consistency index during last day of storage. This showed the close link of the interaction of organic acids of postbiotics with the milk components which resulted in variations of consistency index.

The results of samples containing postbiotics of *Lb. plantarum* at 1^{st} day of storage were similar to the results observed by Nemska et al. (2021), for milks fermented with various strains of *Lb. plantarum* (strains BS41, S8, S9, and S10). As reported by Vélez-Ruiz & Barbosa-Cánovas (1998), the increased consistency index (K) of beverages is often desirable and is associated with increased sensory attributes among consumers. If above-mentioned situation is considered, it is assumed that samples having postbiotics of *Lb.*

plantarum, *Lb. paraplantarum*, and *Lc. lactis* showed higher rheological attributes at the end of 10-day storage.

For all the samples the value of "n" was less then '1' (Table 4.12) which showed the shear thinning and pseudoplastic behavior. Here, with increasing stress the strain increases in a non-linear way (Khetra, Kisan, Ganguly, & Hussain). The results observed for flow behavior index (n) of all samples were somewhat comparable to values described by Beitane & Ciprovica (2012). The mentioned study also showed the n value in the range of 0.20 to 0.80 after storage. Sample with the addition of postbiotics from *Lb. paraplantarum* showed similar behavior with control sample at day 10 of storage,

For samples having postbiotics from *Lb. brevis*, *E. faecalis* and *E. faecium,* the flow behavior index (n) sharply increased to on day-10, but the values were less than 1 which showed their newtonian behavior. This value was only slightly changed for other samples during storage. The values of flow behavior index (n) were comparable to the findings of Castro et al. (2013) who showed that flow behavior index (n) ranges between 0.4 – 0.8 for probiotic beverages having *Lb. acidophilus* and the variations occur only due to the changing percentage of whey to the beverage.

Table 4.9 Rheological paraments of pasteurized milks having various postbiotics

Sample	Parameter					
	Consistency Index (Pa.s)	Flow Behavior Index	Consistency Index (Pa.s)	Flow Behavior Index	Consistency Index (Pa.s)	Flow Behavior Index
	1		5		10	
Control	0.213 ± 0.00^{A}	0.46 ± 0.00^{B}	0.866 ± 0.00^{A}	0.20 ± 0.00^{B}	0.482 ± 0.00^{B}	0.34 ± 0.00^{A}
E. faecalis	0.640 ± 0.04^{A}	0.27 ± 0.01^{B}	0.525 ± 0.15^{A}	0.32 ± 0.04^{B}	0.185 ± 0.18^{B}	0.60 ± 0.22^{A}
E. faecium	0.485 ± 0.03^{A}	0.34 ± 0.00^{B}	0.527 ± 0.10^{A}	0.31 ± 0.02^{B}	0.132 ± 0.05^{B}	0.59 ± 0.04^{A}
***St. thermophilus* 1-K$_4$**	0.546 ± 0.12^{A}	0.31 ± 0.05^{B}	0.631 ± 0.09^{A}	0.28 ± 0.03^{B}	0.137 ± 0.03^{B}	0.57 ± 0.06^{A}
Lc. lactis	0.410 ± 0.00^{A}	0.38 ± 0.00^{B}	0.440 ± 0.15^{A}	0.35 ± 0.07^{B}	0.328 ± 0.31^{B}	0.47 ± 0.24^{A}
Lb. plantarum	0.639 ± 0.12^{A}	0.28 ± 0.05^{B}	0.503 ± 0.14^{A}	0.33 ± 0.04^{B}	0.397 ± 0.43^{B}	0.46 ± 0.29^{A}
Lb. paraplantarum	0.623 ± 0.09^{A}	0.30 ± 0.04^{B}	0.559 ± 0.14^{A}	0.30 ± 0.05^{B}	0.467 ± 0.10^{B}	0.35 ± 0.04^{A}
Lb. brevis	0.618 ± 0.11^{A}	0.29 ± 0.04^{B}	0.298 ± 0.39^{A}	0.48 ± 0.25^{B}	0.028 ± 0.01^{B}	0.80 ± 0.07^{A}
Lb. coryniformis	0.576 ± 0.06^{A}	0.31 ± 0.02^{B}	0.405 ± 0.31^{A}	0.38 ± 0.14^{B}	0.316 ± 0.43^{B}	0.51 ± 0.34^{A}
***Lb. helveticus* 9-B$_5$**	0.612 ± 0.08^{A}	0.29 ± 0.03^{B}	0.829 ± 0.10^{A}	0.21 ± 0.03^{B}	0.205 ± 0.04^{B}	0.52 ± 0.05^{A}
Lb. bulgaricus	0.499 ± 0.17^{A}	0.31 ± 0.03^{B}	0.510 ± 0.12^{A}	0.32 ± 0.05^{B}	0.289 ± 0.37^{B}	0.50 ± 0.25^{A}

The differences ($P < 0.05$) between storage time were indicated with upper-case letters (A, B). Values are shown as Mean ± SD.

4.3.5 Color analysis

Color is one of the important parameters that sometimes even indirectly describe the quality of milk. Color parameter is easily varied because of many factors including animal feed, intensity of heat treatment etc. it is often measured using three main color spaces. These are the degree of Lightness indicated by "L*", degree of redness indicated by "a*" and degree of yellowness indicated by "b*". Degree of Lightness gives information regarding level of lightness of darkness, and ranges from 0 to 100, a* shows the level of redness of greenness whereas b* shows the degree of yellowness.

Table 4.10 Color index of pasteurized milk during storage

Sample	L*	a*	b*
Control	78 ± 0.00	-2.3 ± 0.00	4.3 ± 0.00[a]
E. faecalis	77.9 ± 0.07	-2.3 ± 0.00	3.60 ± 0.14[a]
E. faecium	78.3 ± 0.35	-1.85 ± 0.07	2.25 ± 0.21[b]
***St. thermophilus* 1-K$_4$**	78.1 ± 0.14	-2.2 ± 0.14	3.95 ± 0.07[a]
Lc. lactis	77.8 ± 0.49	-2.35 ± 0.07	3.50 ± 0.71[ab]
Lb. plantarum	77.7 ± 0.42	-2.15 ± 0.07	3.55 ± 0.21[ab]
Lb. paraplantarum	78 ± 0.00	-2.2 ± 0.14	3.75 ± 0.07[a]
Lb. brevis	77.9 ± 0.57	-2.25 ± 0.35	4.35 ± 0.07[a]
Lb. coryniformis	77.9 ± 0.14	-2.15± 0.07	3.70 ± 0.14[a]
***Lb. helveticus* 9-B$_5$**	78 ± 0.14	-2.3 ± 0.42	4.35 ± 0.78[a]
Lb. bulgaricus	77.9 ± 0.07	-2.15 ± 0.07	3.90 ± 0.00[a]

Values are shown as Mean ± S.D (n=11). Significant difference was only found among values of degree of lightness b* (P>0.05).

No significant difference were observed both among samples and among storage periods (P>0.05). Moreover, color index values obtained for the samples with different postbiotics showed a similar trend with the control sample. Postbiotics from *E. faecium* showed maximum lightness level of 78.3 while with a less difference, postbiotics from *Lb. plantarum* showed lightness level of 77.7.

The negative values for the degree of redness indicated the continuous trend towards greenness. This might be largely effected by the initial color of milk before pasteurization as control also showed the same trend.

4.4 Microbiological Analyses

All postbiotics showed intense antimicrobial role against pathogen *E. coli* (ATCC 25922), hence *E. coli* (ATCC 25922) was taken as the test pathogen for estimating bioprotective role of these postbiotics.

The microbiological analysis was done to find out the bio-preservative capacity of various postbiotics against pathogen *E. coli* and their bio-protective role to enhance the shelf life of pasteurized milk. Pasteurized milk samples containing postbiotics were tested against negative control (containing only pasteurized milk) and positive control (containing *E. coli*) and log reduction was tested during the storage days. The microbiological testing was observed separately on BHIA and PCA to observe *E. coli* and TAMB count, respectively.

4.4.1 *E. coli* count

The following table (Table 4.11) shows the log reduction in *E.coli* on day 1, 5 and 10 of storage period. The samples containing postbiotics were tested on BHIA agar and kept for incubation to observe *E. coli* colonies formed or suppressed during storage period.

Table 4.11 *E. coli* count (log CFU/ mL) during the storage of pasteurized milk

Sample	**Storage time**		
	Day 1	**Day 5**	**Day 10**
Positive control (*E. coli*)	6.46 ± 0.00^{aA}	6.65 ± 0.00^{aAB}	6.61 ± 0.21^{aB}
E. faecalis	5.45 ± 0.64^{abA}	5.20 ± 0.28^{abAB}	5.15 ± 0.21^{abB}
E. faecium	6.35 ± 0.20^{bA}	5.57 ± 0.81^{bAB}	2.50 ± 3.54^{bB}
***St. thermophilus* 1-K$_4$**	5.80 ± 0.14^{abA}	5.35 ± 0.07^{abAB}	5.35 ± 0.49^{abB}
Lc. lactis	6.39 ± 0.01^{abA}	5.28 ± 0.03^{abAB}	5.30 ± 0.43^{abB}
Lb. plantarum	6.19 ± 0.21^{abA}	6.27 ± 0.05^{abAB}	5.81 ± 0.43^{abB}
Lb. paraplantarum	6.02 ± 0.34^{abA}	6.04 ± 0.37^{abAB}	5.35 ± 0.49^{abB}
Lb. brevis	5.97 ± 0.24^{abA}	5.59 ± 0.83^{abAB}	5.00 ± 0.00^{abB}
Lb. coryniformis	5.57 ± 0.81^{abA}	5.30 ± 0.43^{abAB}	5.00 ± 0.00^{abB}
***Lb. helveticus* 9-B$_5$**	5.96 ± 0.51^{abA}	5.30 ± 0.43^{abAB}	4.99 ± 0.69^{abB}
Lb. bulgaricus	6.18 ± 0.04^{abA}	5.80 ± 0.14^{abAB}	5.35 ± 0.49^{abB}

The differences ($P < 0.05$) among all samples were indicated with lower-case letters (a, b, ab etc.) while the differences between storage time were indicated with upper-case letters (A, B). Values are shown as Mean ± SD.

Since the negative control was prepared without pathogen (*E. coli*) hence, no growth of pathogen was observed during and after 10 days of storage. Positive control showed a significant increase in *E. coli* starting from 6.46 to 6.65 on day 5. Among Enterococcus species, a sharp decline in pathogen was observed for samples containing postbiotics of *E. faecium*. This was evident from comparison of sample at day 1 (6.35) to the 2.50 log reduction at 10th day. Samples with postbiotics from *E. faecalis* also showed log reduction during 10-day storage.

Sample having postbiotic from *St. thermophilus* 1-K$_4$ showed no significant decrease in pathogen colony after day 5 but the results were statistically similar to that of samples containing *E. faecium* postbiotics. Limited antimicrobial activity was shown by some strains of *St. thermophilus* (Taj et al., 2022). Postbiotics obtained from *Lc. lactis* also affected the pathogen population as the log reduction was seen from 6.39 at day-1 to 5.30 at day-10. Although a slight increase was observed from day 5 but it was not significant.

Similar antimicrobial activity by CFS obtained from another strain of *Lc. lactis* (ATCC-11454) was also observed by Millette, Smoragiewicz, & Lacroix (2004).

Among Lactobacillus species, although all samples showed decrease in *E. coli* colonies after 10-day storage, but it was seen that samples containing postbiotics from *Lb. brevis*, *Lb. helveticus* 9-B_5, and *Lb. bulgaricus* showed significant high log reduction in pathogen during storage. For sample containing postbiotics of *Lb. bulgaricus*, the decrease was evident from 6.18 to 5.35 at last day. The decrease in pathogen could be attributed to the presence of many bacteriocin like metabolites present in the postbiotics of LAB (Pujato et al., 2014).

A slight reduction was also observed in samples having postbiotics of *Lb. plantarum* and *Lb. paraplantarum*. This reduced antimicrobial activity of *Lb. plantarum* was also described by Hu et al. (2019), but it was shown by different strains of *Lb. plantarum*. Contrary to other results, the reduction of *E. coli* was not high as compared to other postbiotics. The probable reason could be the presence of some enzymes in milk like proteinase which could counter the antimicrobial activity of postbiotics of *Lb. plantarum*. The initial composition of growth medium for *Lb. plantarum* could also affect its antimicrobial activity. The addition of 5% glycerol in the MRS broth could significantly affect the production of bacteriocin by *Lb. plantarum* Cys5-4 which results in increased amount of antimicrobial activity (Tenea & Barrigas, 2018).

Another possibility is the presence of bacteriocins that are produced by LAB. Bleicher et al. (2010) identified these bacteriocins and observed their inhibition spectrum against gram-negative *E. coli* bacteria. Postbiotics from *Lb. brevis* also showed the antimicrobial activity but in a much smaller percentage. This activity could be comparable to the results obtained by Barzegar et al. (2023), but since much higher percentage of postbiotics were used the antimicrobial effect obtained was higher than our findings. Also, different commodities interact differently in the presence of postbiotics.

4.4.2 TAMB count

TAMB count is necessary to estimate the microbial load in pasteurized milk. It depends on the storage conditions like hygiene and sanitary situations and is sometimes also deemed to be effected by the presence of other pathogens and microbes (Freitas, Nero, & Carvalho, 2009).

Table 4.12 TAMB count (log CFU/ mL) during storage

Sample	**Storage time**		
	1	**5**	**10**
Positive control (*E. coli*)	6.82 ± 0.03^{A}	6.84 ± 0.14^{B}	6.70 ± 0.00^{B}
E. faecalis	6.36 ± 0.18^{A}	5.86 ± 0.78^{B}	6.29 ± 0.02^{B}
E. faecium	6.59 ± 0.15^{A}	5.94 ± 0.33^{B}	5.98 ± 0.54^{B}
***St. thermophilus* 1-K$_4$**	6.39 ± 0.54^{A}	5.94 ± 0.65^{B}	5.63 ± 0.46^{B}
Lc. lactis	6.45 ± 0.08^{A}	6.08 ± 0.54^{B}	5.94 ± 0.09^{B}
Lb. plantarum	6.25 ± 0.11^{A}	5.99 ± 0.30^{B}	6.56 ± 0.30^{B}
Lb. paraplantarum	6.49 ± 0.23^{A}	6.00 ± 0.73^{B}	5.78 ± 0.25^{B}
Lb. brevis	6.48 ± 0.67^{A}	6.18 ± 0.04^{B}	5.83 ± 0.07^{B}
Lb. coryniformis	6.44 ± 0.33^{A}	5.98 ± 0.19^{B}	5.50 ± 0.49^{B}
***Lb. helveticus* 9-B$_5$**	6.41 ± 0.58^{A}	5.74 ± 0.37^{B}	5.74 ± 0.37^{B}
Lb. bulgaricus	5.89 ± 0.40^{A}	5.98 ± 0.04^{B}	6.09 ± 0.12^{B}

The differences between storage time were indicated with upper-case letters (A, B). Values are shown as Mean ± SD.

In the evaluated samples the TAMB counts was observed to be significantly different (P<0.05) during storage durations. The negative control was prepared without any postbiotic and pathogen, hence no bacterial growth was observed in negative control during 10-day storage. However, all samples at day-5 of storage showed similar statistical results with that of day 1 and 10. In the obtained results, we could observe the increase in TAMB count for samples containing postbiotics of *Lb. plantarum* and *Lb. bulgaricus*. This indicates that the presence of postbiotics had a count effect on the thriving ability of mesophilic bacteria. As shown by Ziadi et al. (2019), the increased aerobic mesophilic count in samples having postbiotics of *Lb. plantarum* might happen due the neutralization of organic acids (like lactic acid) and hence, subsequent loss in their antimicrobial activity which cause the TAMB to thrive more easily in the samples. In case of samples having postbiotics of *Lb. bulgaricus*, a slight increase TAMB count could be contributed due to probable contamination during storage as indicated by Edalatian Dovom et al. (2022), who observed high TAMB in traditional fermented milk called Kishk during storage.

The decrease in TAMB count during storage, in samples containing postbiotics of *Lc. lactic* could be explained by the presence of various strain-dependent bacteriocins as the results shown in table 4.15. The results are contrary to the findings of Kaya & Şimşek (2020) for *L. lactis* PFC77 strain that showed very little decrease in TAMB during fermentation.

The increase in aerobic mesophilic bacteria is contrary to the findings of Barzegar et al. (2023), who showed significant decrease in bacterial load by addition of lactobacillus postbiotics in various edible coatings.

5. CONCLUSION

Although probiotics have been in use for quite some time, the concept of using postbiotics or bacterial metabolites is relatively new. Upon determining the effectiveness of beneficial bacteria and some negative effects associated with their use in immune-deficient individuals, in-depth studies have begun to develop alternative applications. In dairy industry, the challenges associated with the presence of live bacteria prevent some manufacturers from using them in dairy products, as any uncertain changes during storage might intervene with the product and can cause sensory defects. It is because live bacteria are organisms which continuously reproduce when exposed to optimal temperature and pH range. This continuous activity results in changes in rheology and viscosity as well as cause serious sensory deterioration especially like LAB which produce organic acids.

Against the above-mentioned challenges, postbiotics are deemed to be considered as an effective alternative. Postbiotics possess the same metabolites as those produced by the beneficial bacteria and it could be assumed that the positive effects of postbiotics will be delivered in a similar way. In the current study, the antioxidant activity, phenolic contents, organic acid profile and antimicrobial activity of postbiotics obtained from 10 different indigenous LAB species were evaluated. The trends observed during these analyses are summarized in the Table 5.1.

In terms of phenolic content, the highest amount was produced by postbiotics of *Lb. paraplantarum* that was 0.71 ± 0.18 mg GAE/ mL. In addition, postbiotics of *E. faecalis* and *Lb. helveticus* 9-B_5 were also capable of producing adequate quantities of phenolic contents i.e., 0.66 ± 0.24 and 0.66 ± 0.14 respectively. According to the antioxidant activities, the highest antioxidant activity was shown by postbiotics from *Lb. plantarum, E. faecalis* and *Lc. lactis* that was ranging as 3.67 ± 0.25, 3.60 ± 0.11 and 3.58 ± 0.18, respectively. At the same time, the results showed that the lowest antioxidant potential was possessed by postbiotics of *Lb. bulgaricus, Lb. coryniformis, Lb. helveticus* 9-B_5. For antimicrobial activity, six pathogen bacteria were evaluated during analyses. According to the obtained results, postbiotics from *E. faecalis*, *Lb. paraplantarum* and *Lb. coryniformis* showed antimicrobial activity among maximum number of pathogens, followed by postbiotics of *E. faecium*, *Lb. plantarum* and *Lc. lactis*. Most postbiotics were effective against *E. coli* while no anti-microbial activity was shown against *B. cereus* and *S. aureus*.

The organic acid profile of all postbiotics was determined by HPLC and all postbiotics showed varying amounts of organic acids. Some organic acids were produced in minimum concentrations e.g., butyric acid. Butyric acid was not detected in postbiotics of Enterococcus species and *Streptococcus thermophilus* 1-K_4. It was also present in extremely low amount in other postbiotics. Contrary to above, Lactic acid and Acetic acid was produced in generous amounts by all postbiotics. Postbiotics from *Lb. helveticus* 9-B_5, *Lb. coryniformis* and *Lb. bulgaricus* were significantly high in lactic acid concentrations (21368.32 mg/g, 19489.06 mg/g and 19400.28 mg/g, respectively). Postbiotics of *Lb. coryniformis* also had higher quantities of acetic acid (56230.19 mg/g). Acetic acid was highest in postbiotics of *Lb. brevis* (56853.01 mg/g), followed by that of *Lb. paraplantarum* (55159.92 mg/g). Acetoin and diacetyl were also detected in all postbiotics but with varying amounts. Highest acetoin was seen in postbiotics of *Lb. paraplantarum* (20722.50 mg/g) followed by that of *Lb. helveticus* 9-B_5 (17481.30 mg/g). Diacetyl was observed to be significantly present in the postbiotics of *Lb. paraplantarum*, followed by that of *Lb. plantarum* and *Lb. brevis*.

To determine the biopreservative role of postbiotics, each postbiotic was individually integrated to pasteurized milk and was analyzed in terms of physicochemical, microbiological, and rheological properties during shelf-life. pH values of pasteurized milk showed strong interaction between sample and storage time ($P < 0.05$). Compared to control, all samples showed a slight decrease in pH on the first day of storage. The decreasing trend was minimum at 5^{th} day but was evident for sample containing postbiotics *E. faecium*. On the 10^{th} day of storage, pH was decreased significantly for some samples especially those containing postbiotics of *Lc. lactis*, and *E. faecium*. This decrease made these postbiotics inefficient for use in pasteurized milk because the significant decrease results in loss of milk quality and could cause aggregation of milk proteins if the trend continues. Variation in pH at 10^{th} day of storage was minimum for samples containing postbiotics from *E. faecalis*, *Lb. plantarum* and *Lb. coryniformis*.

For titratable acidity (%), significant difference (P <0.05) among all samples was detected. Also, there was a significant difference between the storage durations. On the 1st day, all samples showed somewhat similar TA % which was increased to some extent on 5th day. This change was seen in sample having postbiotics from *Lb. paraplantarum*, and *Lb. coryniformis*. The 10th day of storage showed the highest lactic acid percentage (L.A %) in samples having postbiotics of *E. faecium*, *Lc. lactis*, *Lb. helveticus* 9-B_5 and *Lb. bulgaricus*. The TS % showed significant differences both for storage period and for samples (P <0.05). The results showed that TS % was significantly increased on the 5th day of storage and was again decreased on the 10th day.

Table 5.1 Functional and bio-preservative properties of postbiotics *in-vitro* and in pasteurized milk

Sample	Functional properties			Bio preservation in pasteurized milk		
	Antioxidant activity	Phenolic Contents	Anti-microbial activity	Rheology	Anti-microbial activity	Bio-preservative property
E. faecalis	+++	+++	+++	++	+	+
E. faecium	++	+	++	+	+++	++
St. thermophilus 1-K$_4$	++	+	+	+	+	++
L. lactis	+++	++	++	++	++	+++
Lb. plantarum	++	+++	++	++	+	+
Lb. paraplantarum	+	++	+++	+++	+	++
Lb. brevis	++	+	+	+	++	++
Lb. coryniformis	+	++	+++	+++	+	+++
Lb. helveticus 9-B$_5$	+	+++	+	++	++	++
Lb. bulgaricus	+	++	+	+++	++	+

Moderate activity = +; High activity = ++; Very high activity = +++

Postbiotics of *E. faecalis* showed the highest antioxidant activity and phenolic contents among other samples. In pasteurized milk, although it lowered *E. coli* count, the aerobic mesophilic count was increased at 10th day of storage after initially decreasing at 5th day. Hence, it makes it doubtful to be used in pasteurized milk but could be used in other dairy products. Postbiotics of *E. faecium* had significant antioxidant property but lower phenolic contents. Due to its good antimicrobial activity against *E. coli*, it could be utilized in biopreservation of pasteurized milk but the sudden decrease in viscosity as compared to the control could cause a problem.

Postbiotics obtained from *Streptococcus thermophilus* 1-K_4, *Lb. brevis*, *Lb. bulgaricus*, and *Lb. coryniformis* showed minimum to no antimicrobial property *in-vitro* and also their bio-preservative property in pasteurized milk during storage was not significant. Only postbiotics from *Lb. coryniformis* showed decrease in TAMB during storage but had minimum effect against *E. coli* proliferation. *Lb. brevis* decreased the *E. coli* count but significantly increased the TAMB count in milk during 10-day storage.

In samples containing postbiotics of *Lb. plantarum*, although it showed high functional properties but had no antimicrobial activity and shelf-life enhancement ability in pasteurized milk. For postbiotics of *Lb. paraplantarum*, the functional properties, especially antioxidant activity were significantly lower than other postbiotics. The antimicrobial activity was significantly high in *in-vitro*, but this property was not revealed in pasteurized milk thus, no antimicrobial activity and minimum bio preservative ability was seen in the sample.

Postbiotics from *Lb. helveticus* 9-B_5 showed unclear results in terms of functional properties. It had the highest phenolic contents but low antioxidant properties. Apart from that, the postbiotics displayed adequate antimicrobial activity in milk and also decreased the TAMB count. Hence, these could potentially be used in pasteurized milk.

Postbiotics extracted from *Lc. lactis* were significantly high in antioxidants and possessed high phenolic contents. When used in milk, the viscosity was decreased due to decrease in pH, but the reduction was among acceptable levels. The postbiotics exhibit a significant reduction in total aerobic mesophilic count in milk during 10-day storage. Additionally, the reduction in *E. coli* count also showed its antimicrobial activity against the pathogen. Hence, it’s observed that postbiotics from *Lc. lactis* could be used in pasteurized milk as it adequately delivers antimicrobial and bio-preservatory properties in milk.

Future Aspects: Biopreservation of pasteurized milk by adding postbiotics from 10 native LAB species showed the significance of using each of them in milk. Some postbiotics had the additional effect of sudden reduction in the pH level. These could be researched further for their use with other fermented milk beverages like kefir and yogurt where they could provide additional role. Further, the sensory analysis and general acceptability was yet to be evaluated and in future the samples of milk with postbiotics could also be tested for their sensory evaluation. Postbiotics exhibiting higher antimicrobial activity could also be used in dairy products were ageing is required, like hard cheese. Such evaluation of antimicrobial potential in other dairy products would help to enhance to use of these bio-preservative agents in other products as well.

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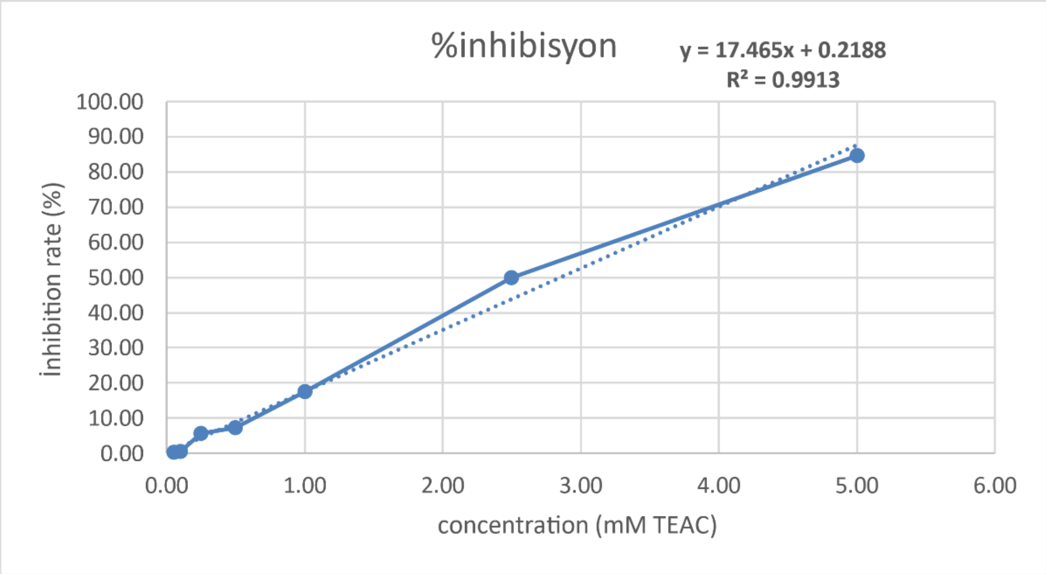
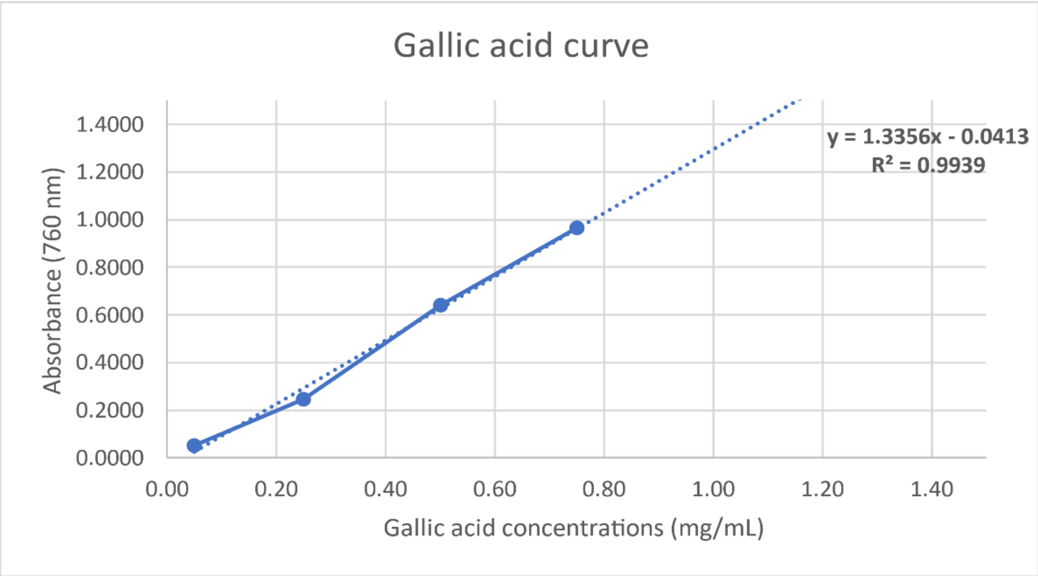
APPENDIX

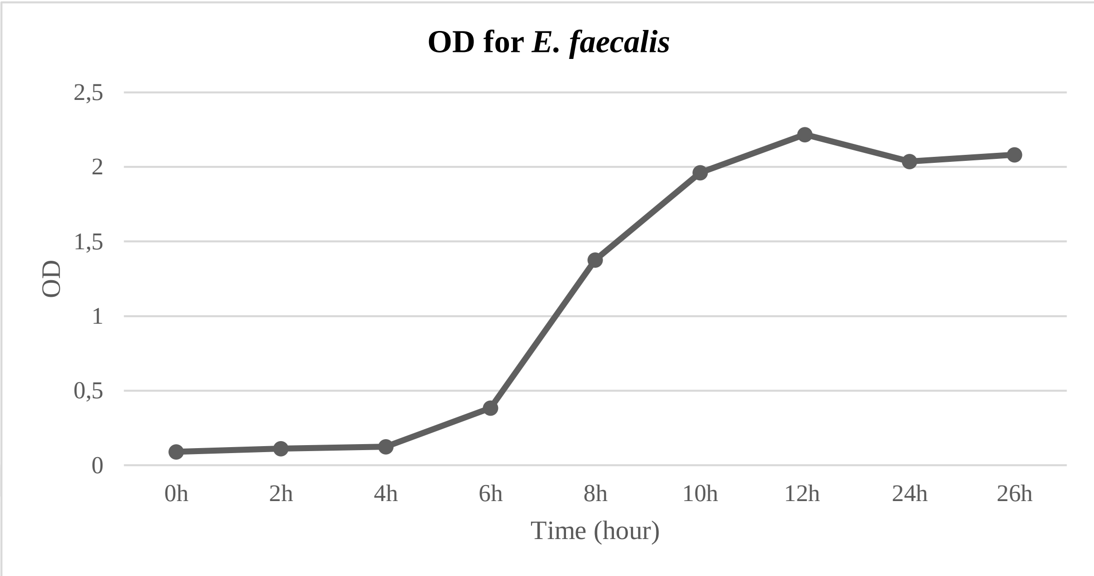
APPENDIX 1 STANDARD CURVE OF TROLOX



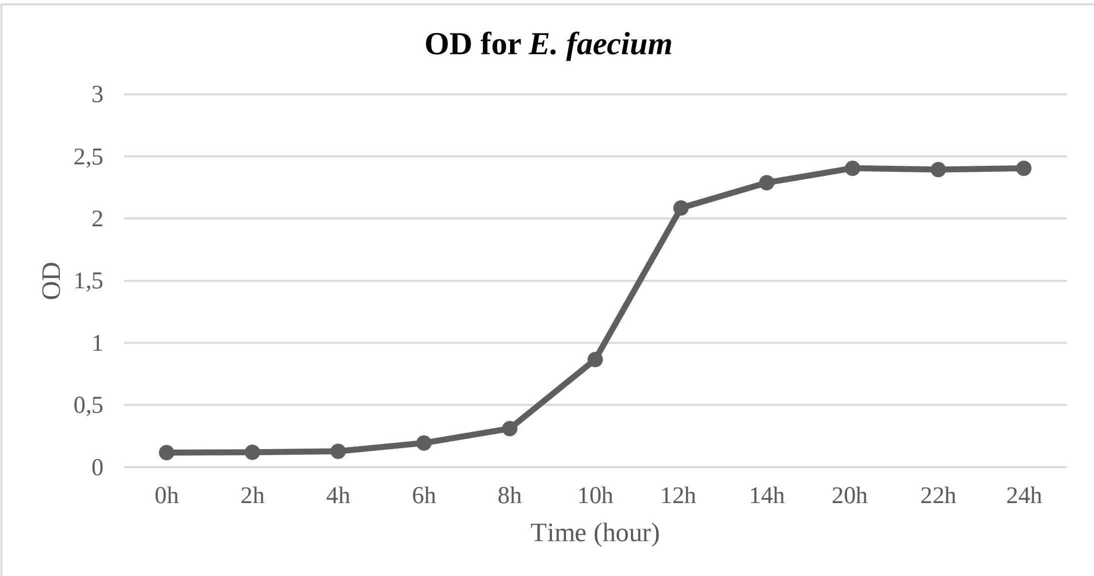
APPENDIX 2 STANDARD CURVE OF GALLIC ACID



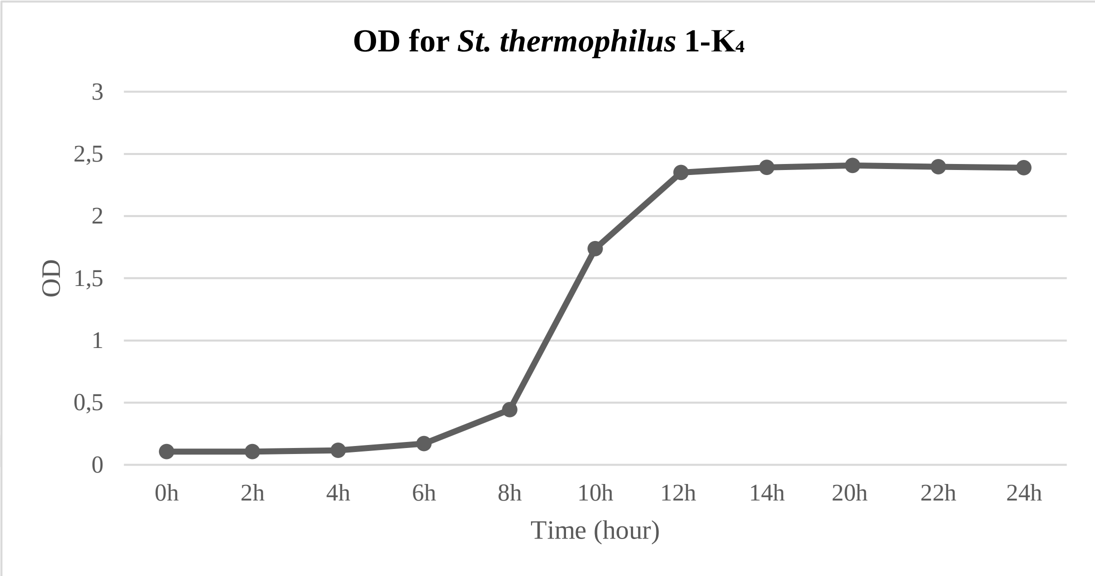
APPENDIX 3 OPTICAL DENSITY REPRESENTATION FOR *E. faecalis*



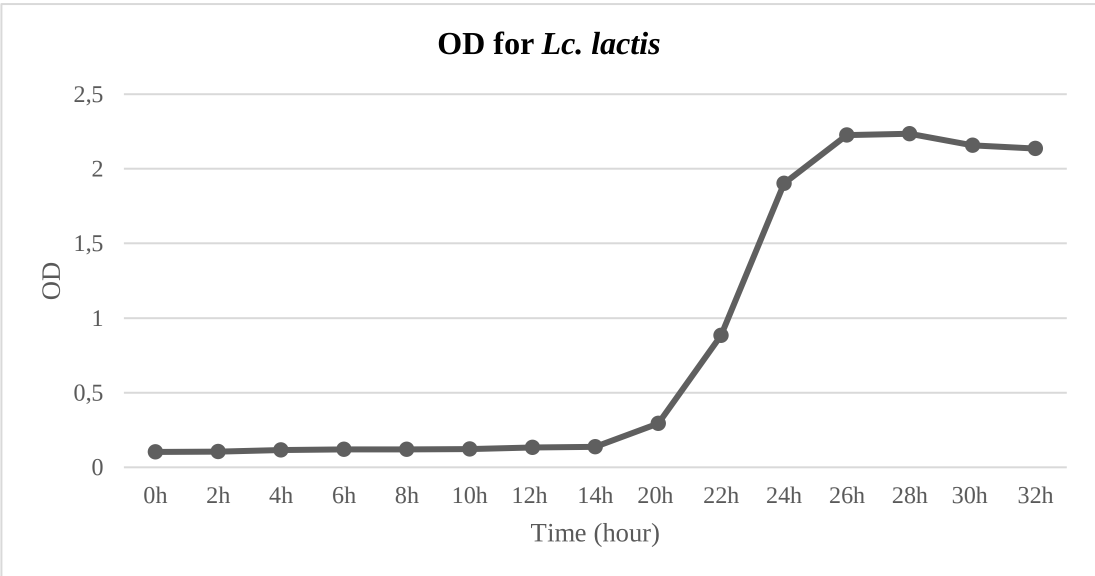
APPENDIX 4 OPTICAL DENSITY REPRESENTATION FOR *E. faecium*



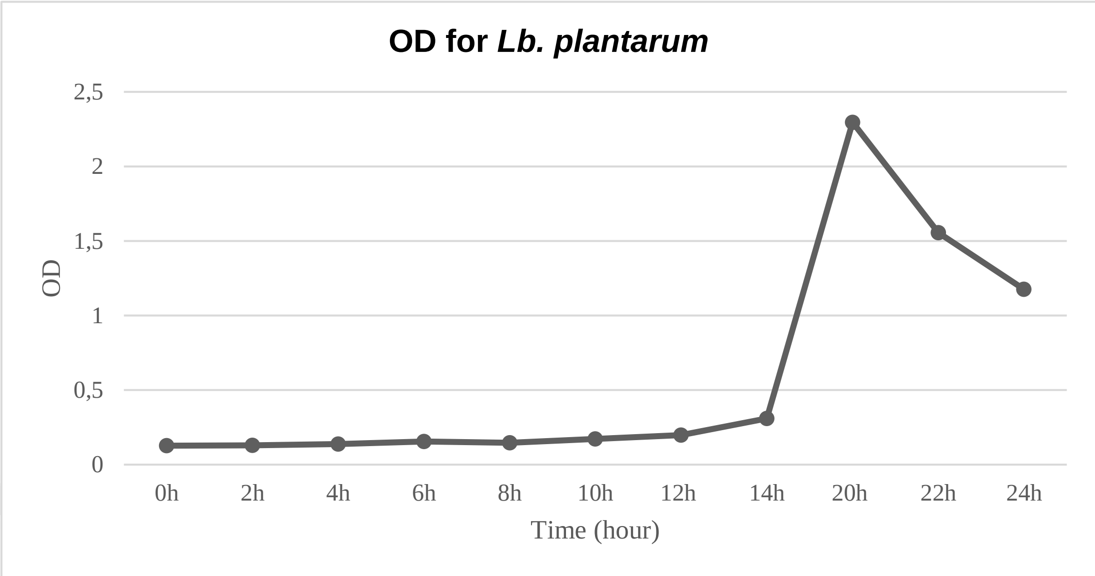
APPENDIX 5 OPTICAL DENSITY REPRESENTATION FOR *St. thermophilus* 1-K_4



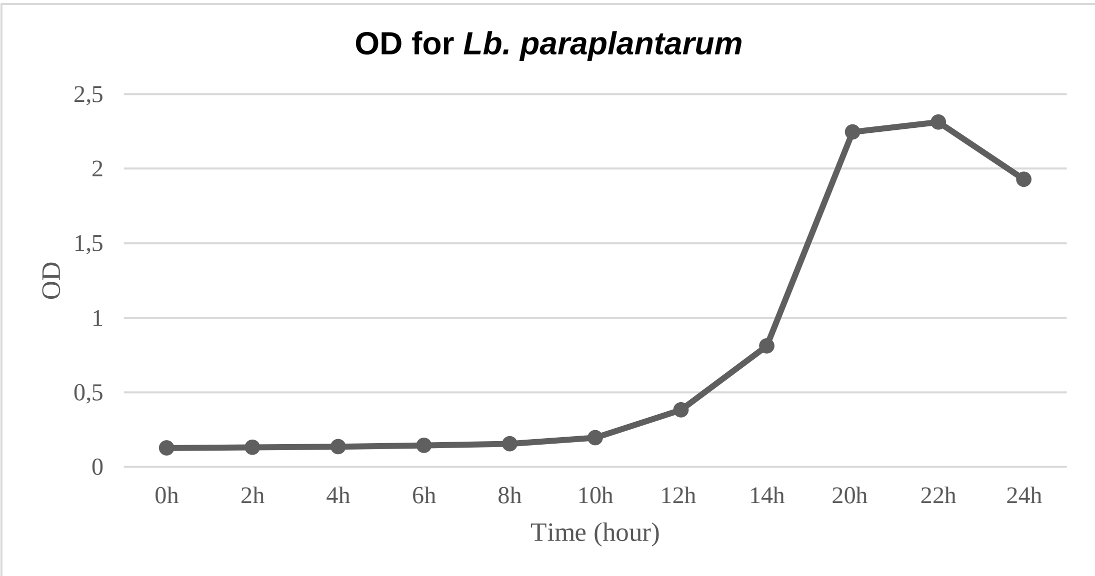
APPENDIX 6 OPTICAL DENSITY REPRESENTATION FOR *Lc. lactis*



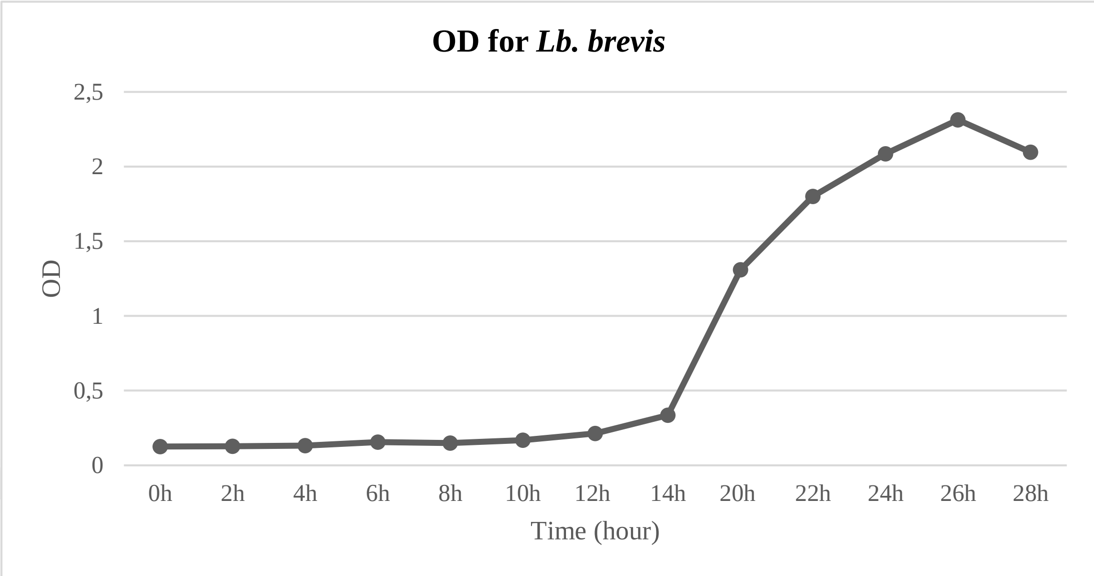
APPENDIX 7 OPTICAL DENSITY REPRESENTATION FOR *Lb. plantarum*



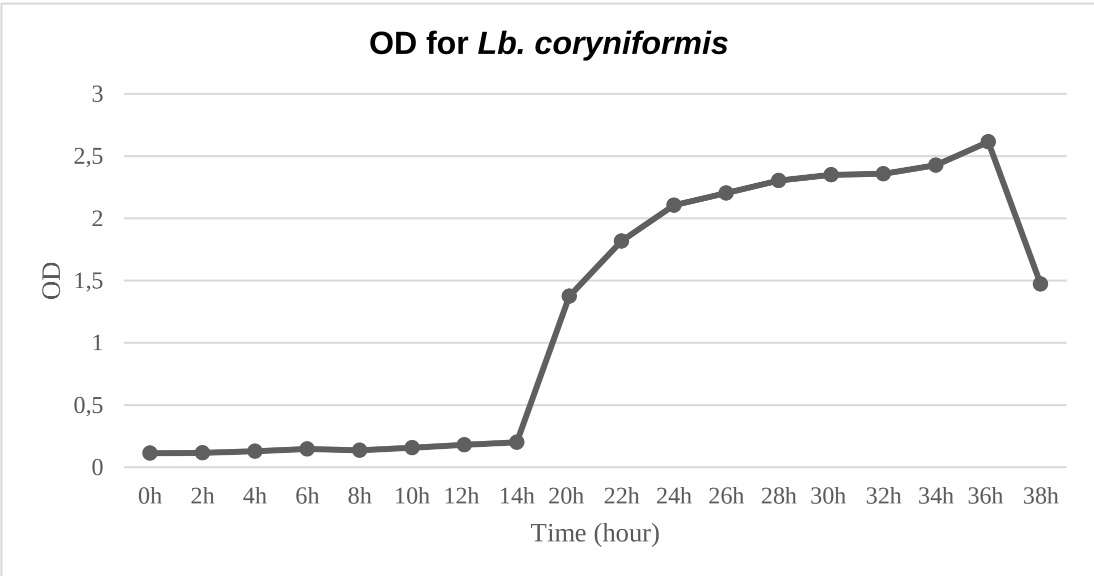
APPENDIX 8 OPTICAL DENSITY REPRESENTATION FOR *Lb. paraplantarum*



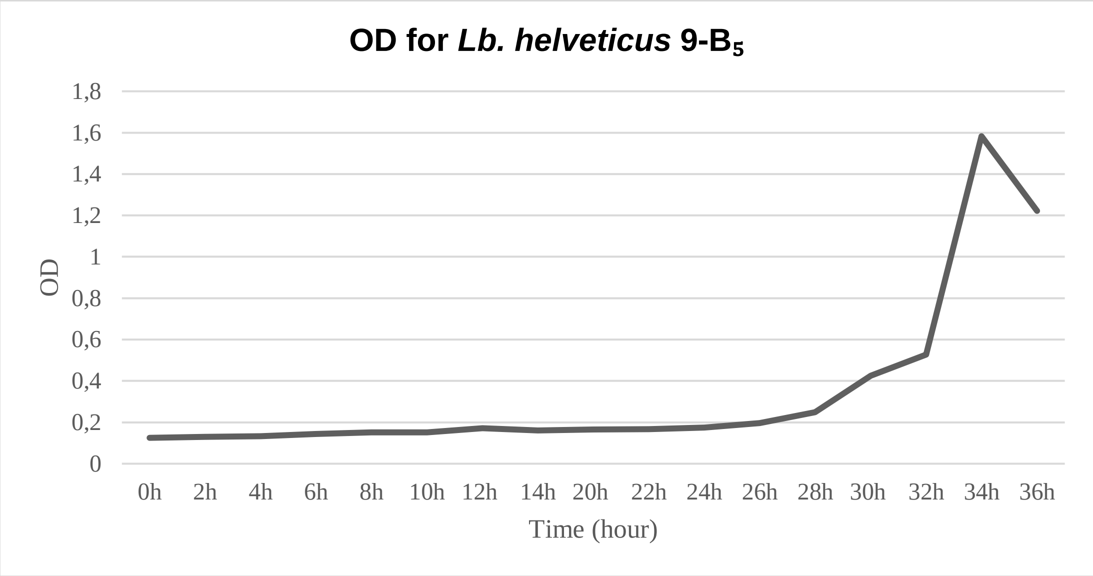
APPENDIX 9 OPTICAL DENSITY REPRESENTATION FOR *Lb. brevis*



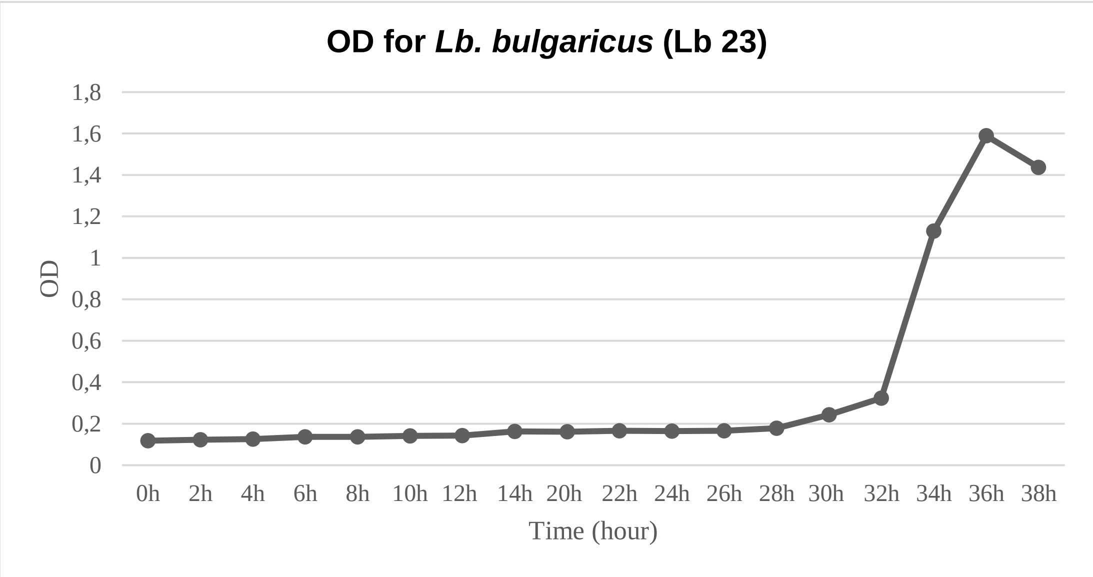
APPENDIX 10 OPTICAL DENSITY REPRESENTATION FOR *Lb. coryniformis*



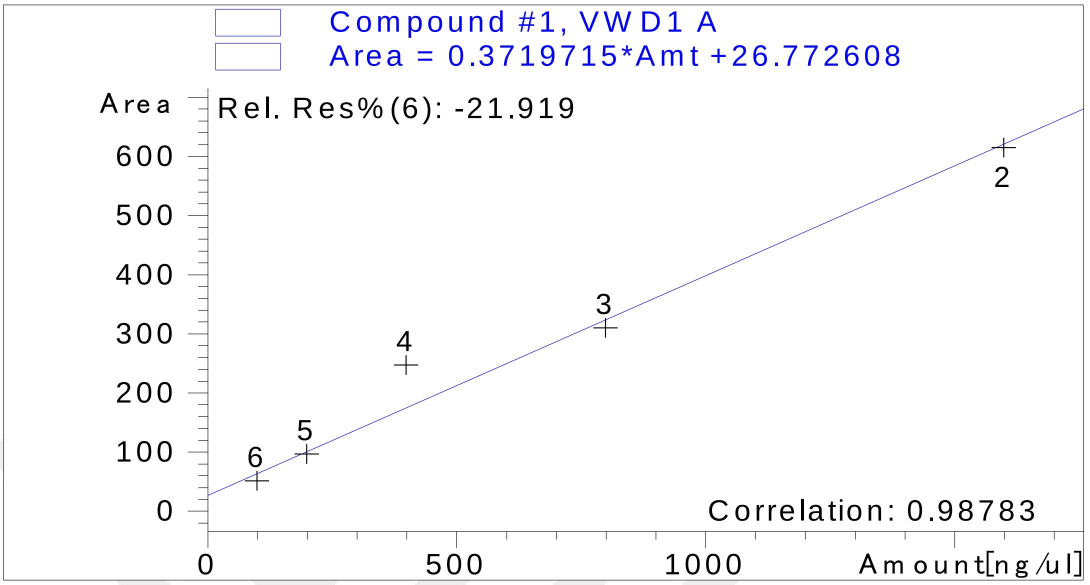
APPENDIX 11 OPTICAL DENSITY REPRESENTATION FOR *Lb. helveticus* 9-B_5



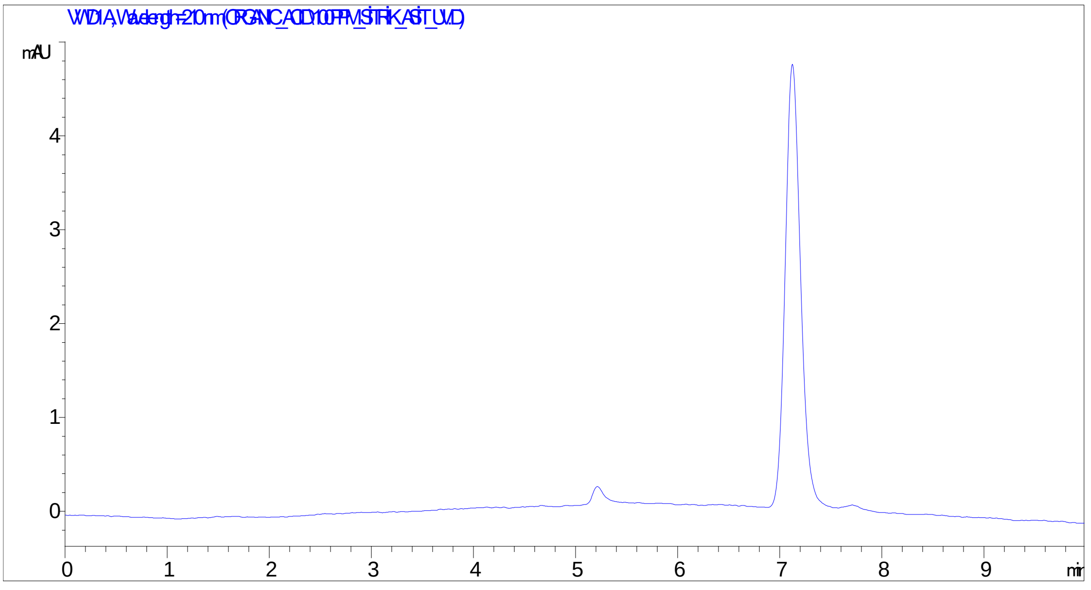
APPENDIX 12 OPTICAL DENSITY REPRESENTATION FOR *Lb. bulgaricus* (LB. 23)



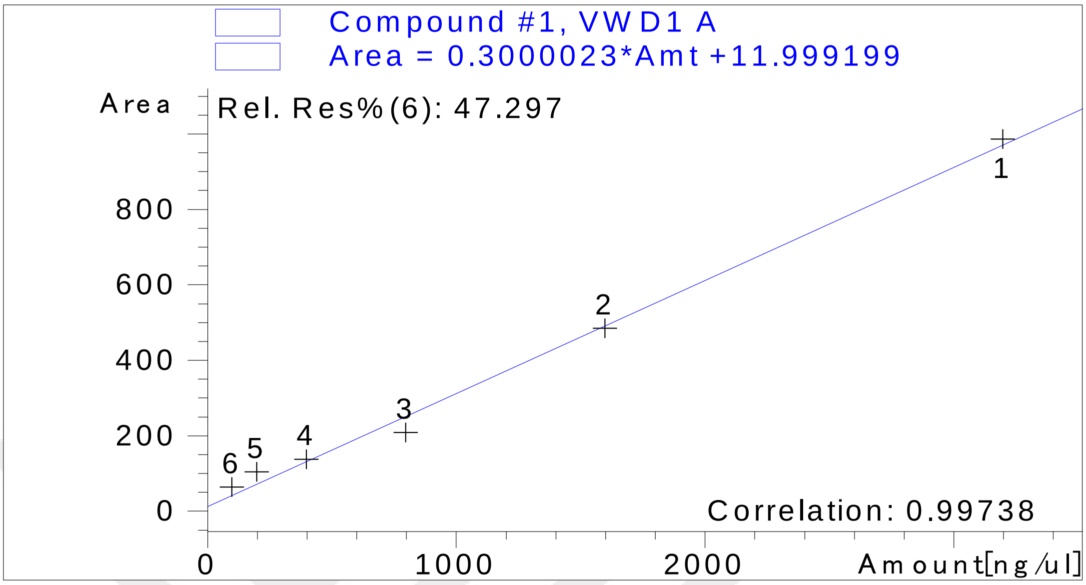
APPENDIX 13 STANDARD CURVE FOR CITRIC ACID



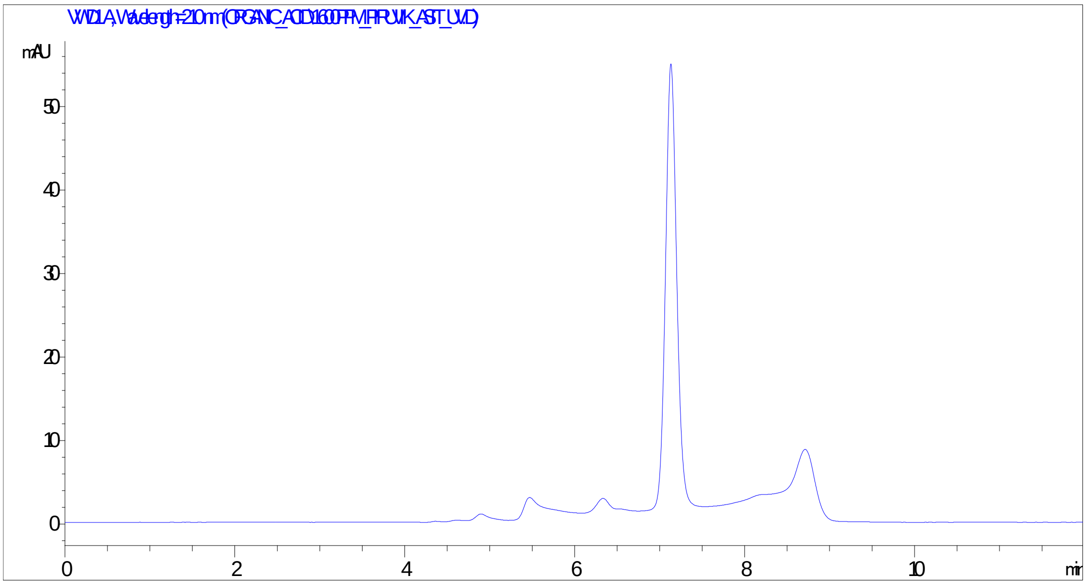
APPENDIX 14 CITRIC ACID PEAK DETECTION AT 100PPM



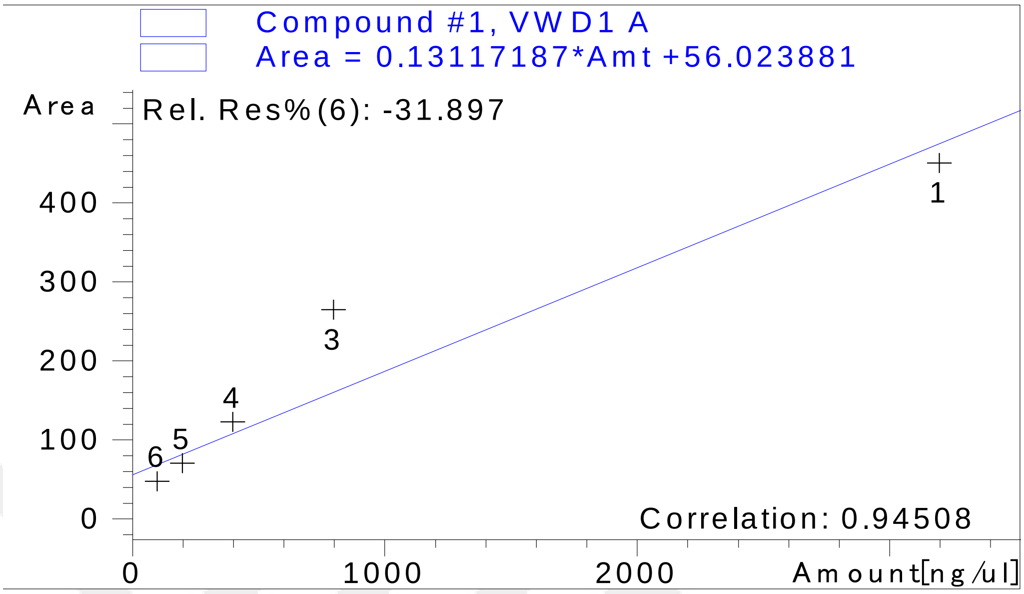
APPENDIX 15 STANDARD CURVE FOR PYRUVIC ACID



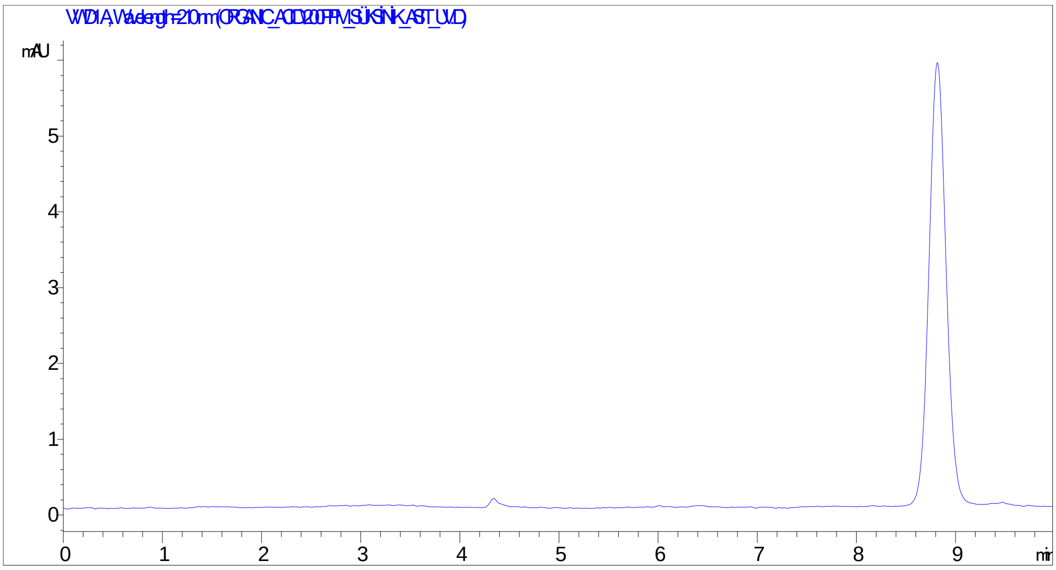
APPENDIX 16 PYRUVIC ACID PEAK DETECTION AT 1600PPM



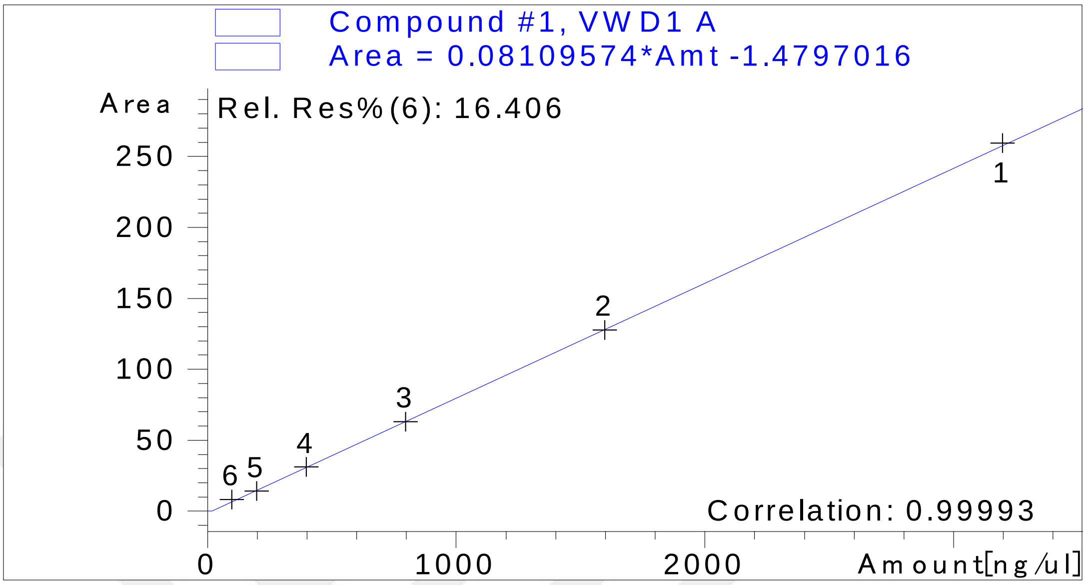
APPENDIX 17 STANDARD CURVE FOR SUCCINIC ACID



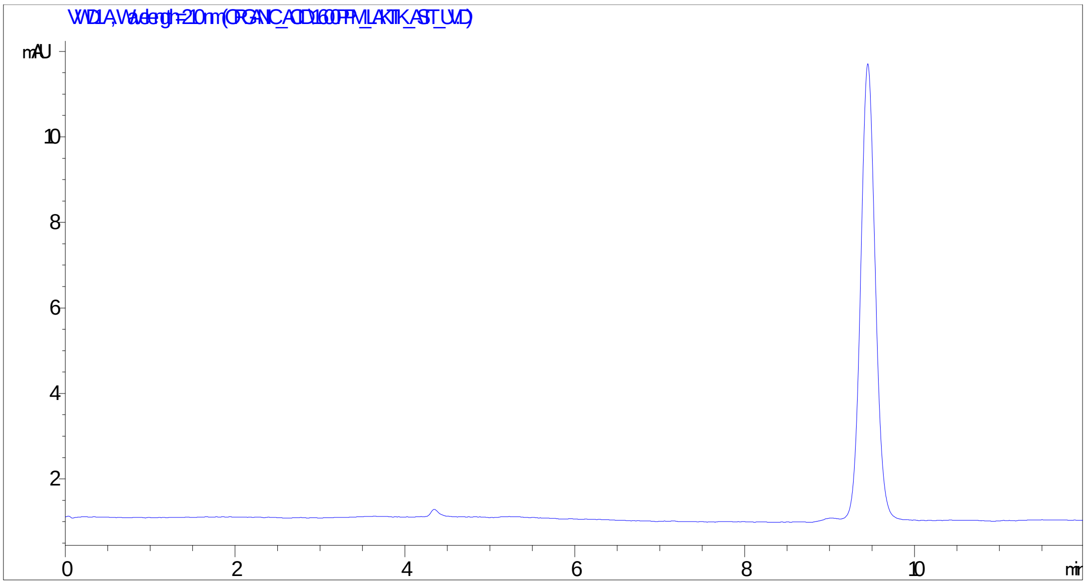
APPENDIX 18 SUCCINIC ACID PEAK DETECTED AT 200NM



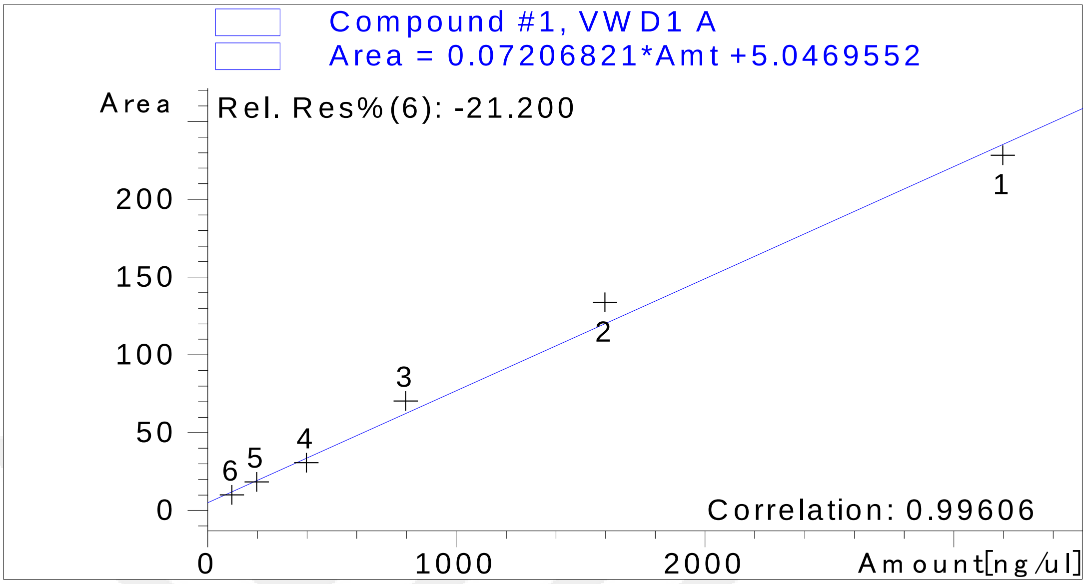
APPENDIX 19 STANDARD CURVE FOR LACTIC ACID



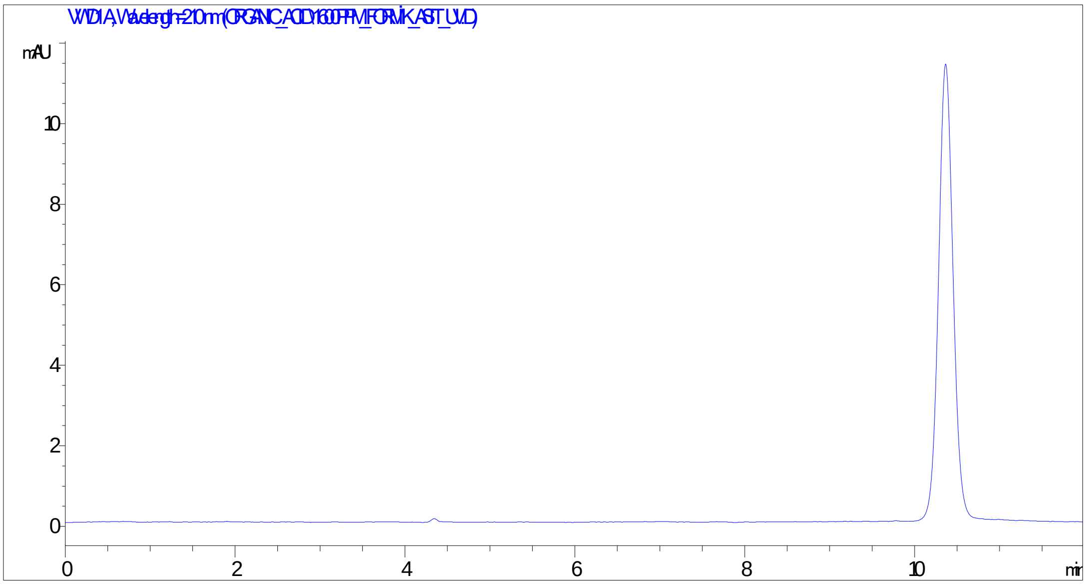
APPENDIX 20 LACTIC ACID CURVE OBSERVED AT 1600PPM



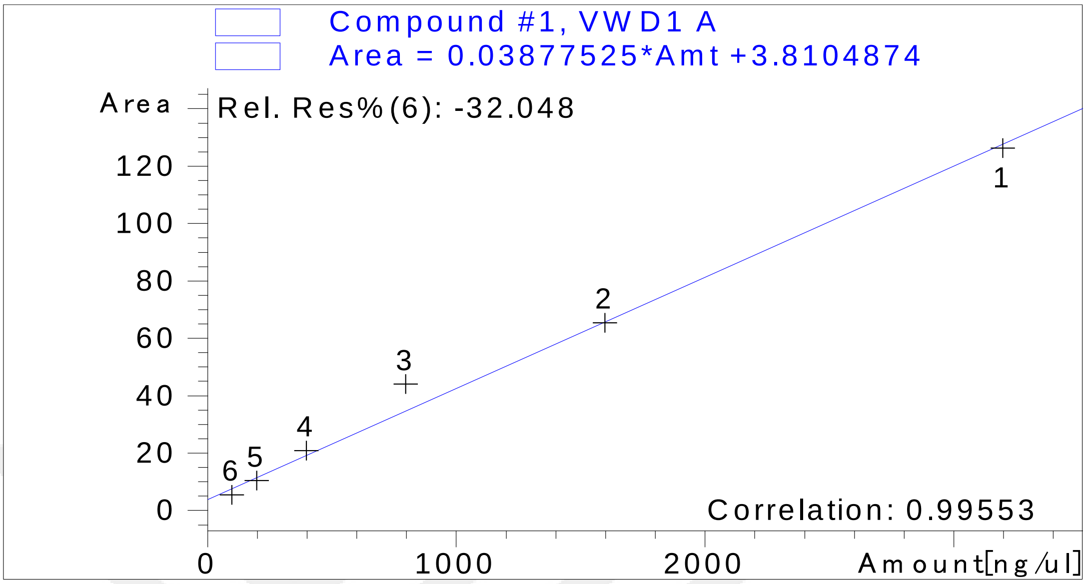
APPENDIX 21 STANDARD CURVE FOR FORMIC ACID



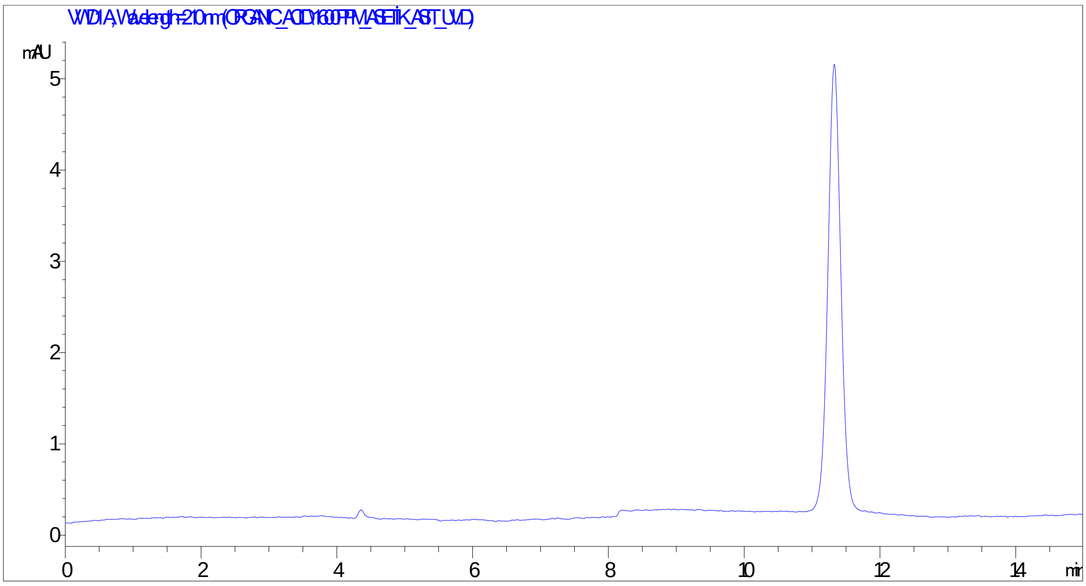
APPENDIX 22 PEAK DETECTION OF FORMIC ACID AT 1600PPM



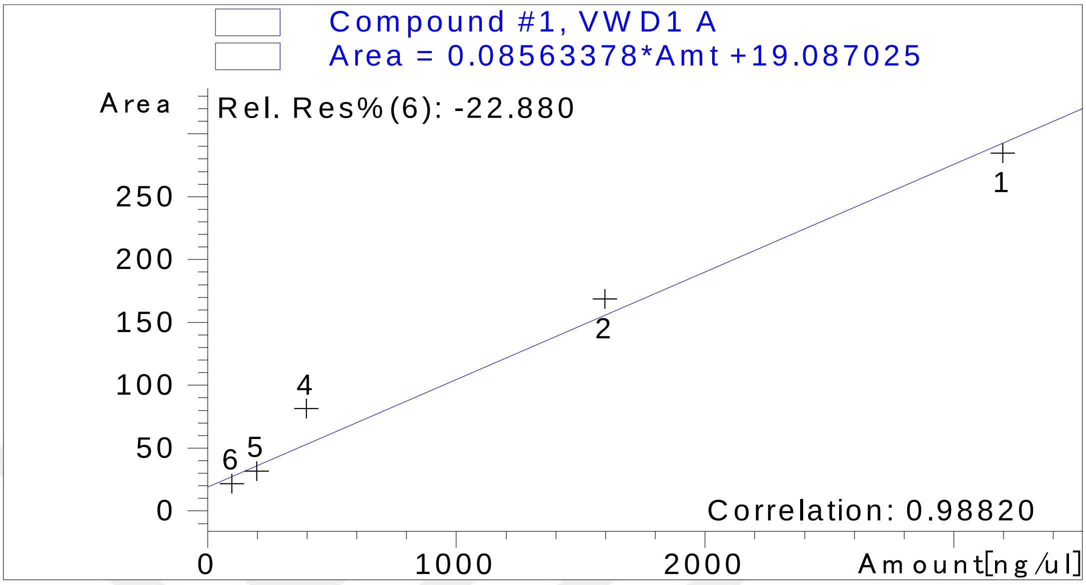
APPENDIX 23 STANDARD CURVE FOR ACETIC ACID



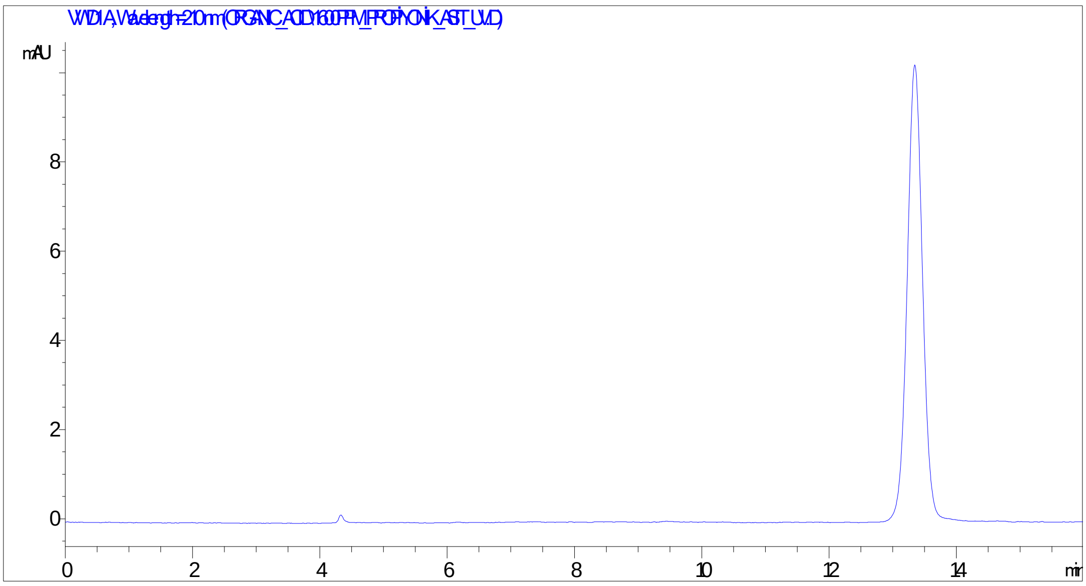
APPENDIX 24 DETECTION OF PEAK FOR ACETIC ACID AT 1600PPM



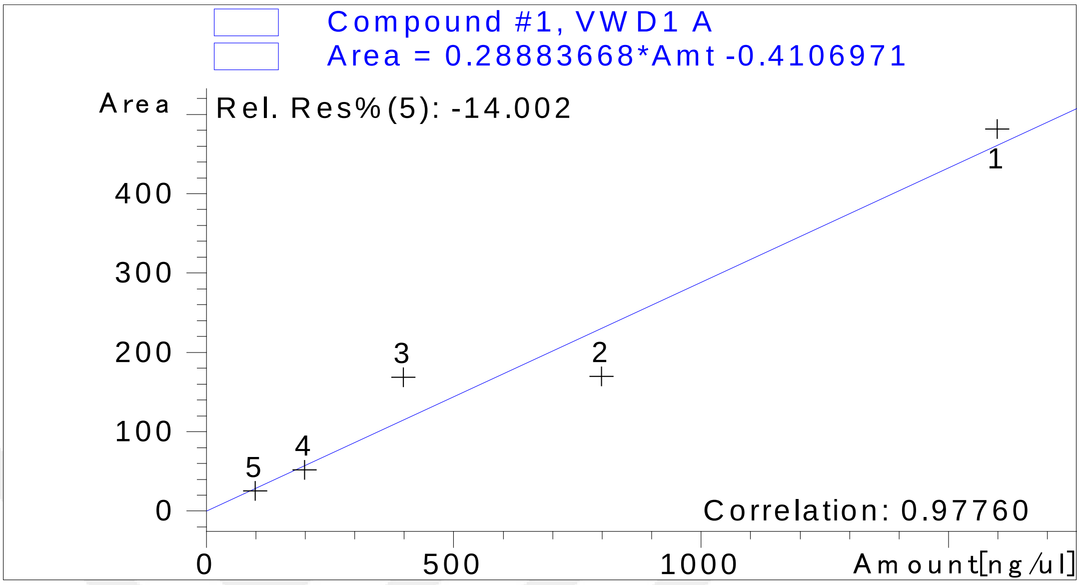
APPENDIX 25 STANDARD CURVE FOR PROPIONIC ACID



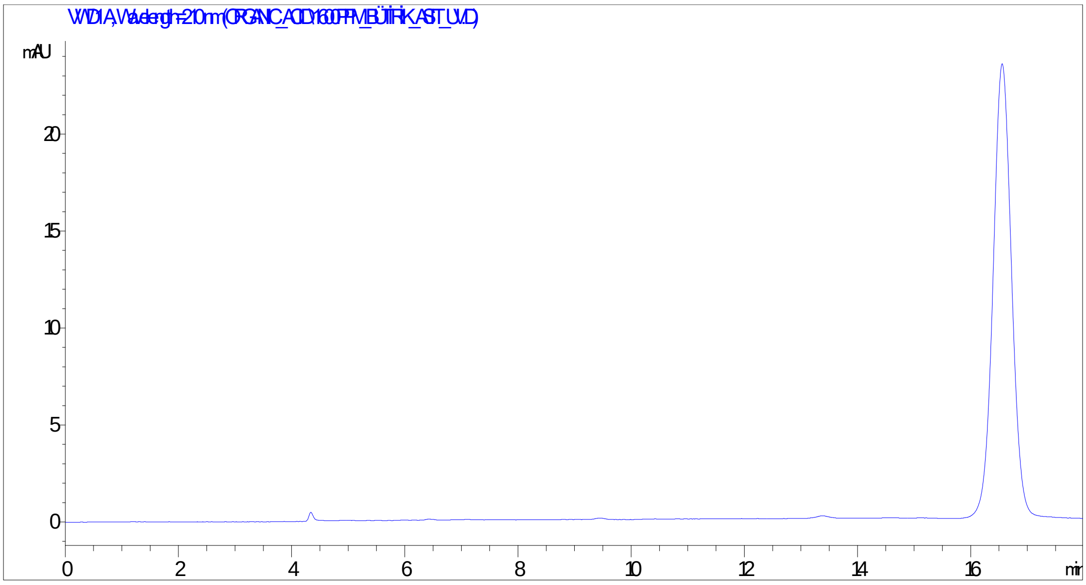
APPENDIX 26 DETECTION PEAK OF PROPIONIC ACID AT 1600PPM



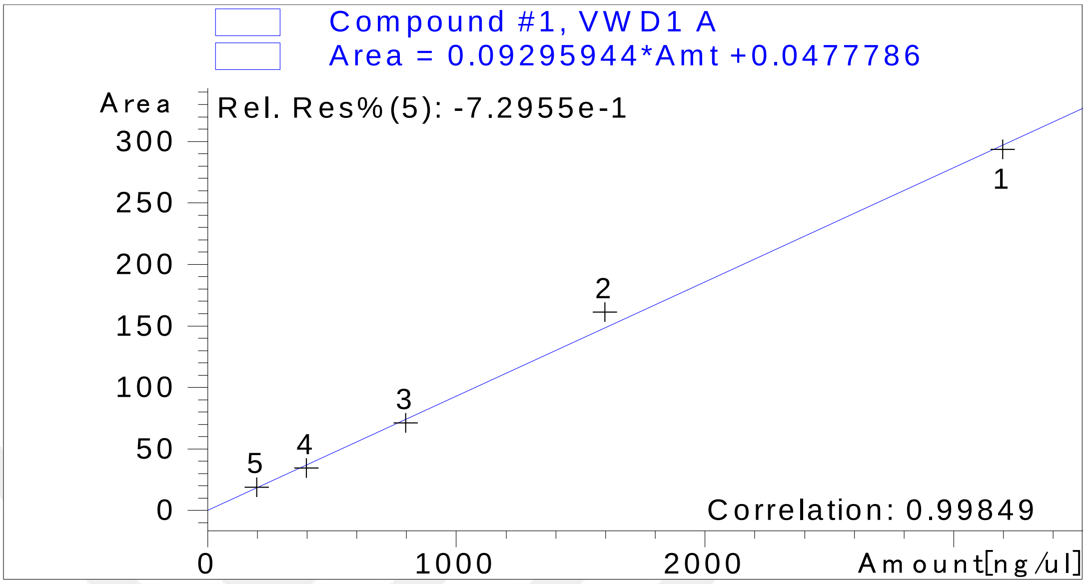
APPENDIX 27 STANDARD CURVE FOR BUTYRIC ACID



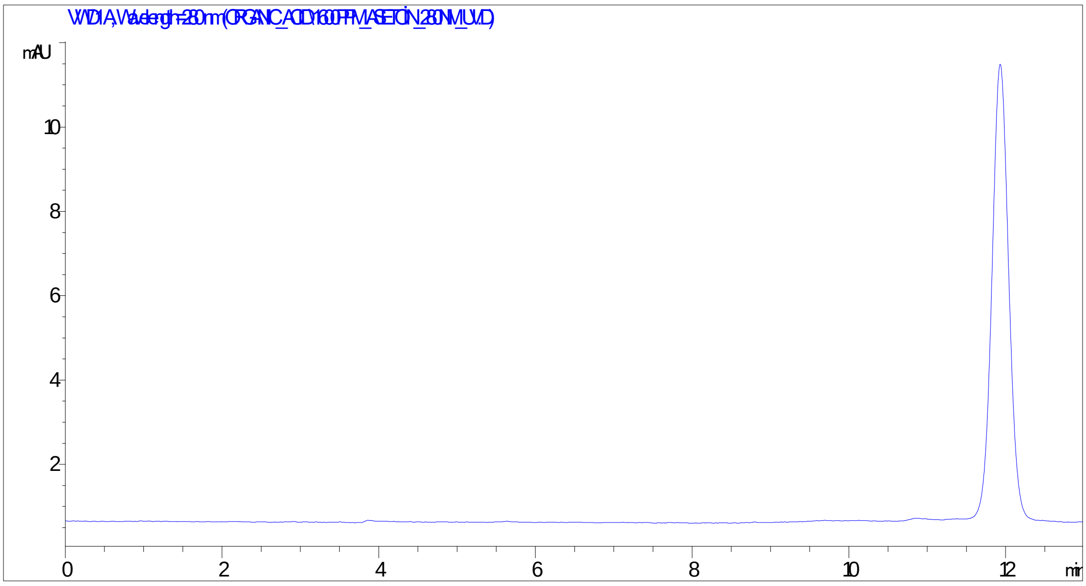
APPENDIX 28 PEAK DETECTION FOR BUTYRIC ACID AT 1600PPM



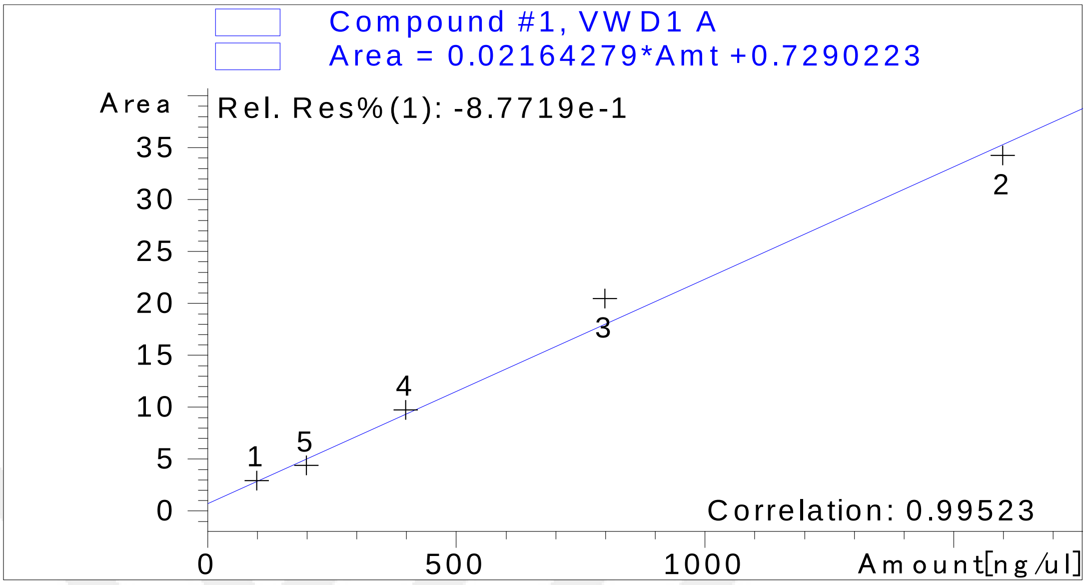
APPENDIX 29 STANDARD CURVE FOR ACETOIN



APPENDIX 30 ACETOIN PEAK DETECTION AT 1600 PPM



APPENDIX 31 STANDARD CURVE FOR DIACETYL



APPENDIX 32 PEAK DETECTION OF DIACETYL AT 200 PPM