# PRODUCTION OF POSTBIOTIC BY LACTIC ACID BACTERIA THROUGH FERMENTATION PROCESS AS POTENTIAL ANTIDIABETIC AGENT

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**by**

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| | |
|---|---|
| LAB | Lactic Acid Bacteria |
| EPS | Exopolysaccharide/ Extracellular Polysaccharide |
| HKC | Heat Killed Cell |
| ATPS | Aqueous Two Phase System |
| DM | Diabetes Mellitus |
| ISAPP | International Scientific Association for Probiotics and Prebiotics |
| GM | Gastrointestinal Microbial |
| SCFA | Short Chain Fatty Acid |
| GABA | Gamma Aminobutaric Acid |
| T1D/M | Type 1 Diabetic/Mellitus |
| T2D/M | Type 2 Diabetic/Mellitus |
| DPP-IV | Dipeptidyl peptidase-IV |
| CS | Culture Cell |
| CPS | Capsular/Cellular Polysaccharide |
| CFS | Cell Free Supernatant |
| CFE | Cell Free Extract |
| IC | Intact Cell |
| BMITF | 1-butyl-3-methylimidazolium tetra-fluoroborate |
| EMIB | 1-ethyl-3-methylimidazolium bromide |
| BMIOS | 1-butyl-3-methylimidazolium octyl sulfate |

**PENGELUARAN POSTBIOTIK OLEH BAKTERIA ASID LAKTIK MELALUI PROSES PENAPAIAN SEBAGAI AGEN ANTIDIABETIK YANG BERPOTENSI**

## ABSTRAK

Sebagai tindak balas kepada kesan buruk kesihatan yang ketara yang berkaitan dengan penggunaan ubat antidiabetik yang disintesis secara kimia untuk individu yang menghidap diabetes, penyiasatan komprehensif telah dijalankan untuk meneroka potensi sebatian postbiotik yang dihasilkan oleh bakteria asid laktik (LAB) sebagai agen antidiabetik baru, dengan tertentu. memberi tumpuan kepada kesan perencatan mereka berbanding acarbose. Tujuh strain LAB telah dikultur untuk mendapatkan supernatan dan sel terbunuh haba (HKC) pada suhu 80℃, yang kemudiannya dinilai untuk aktiviti perencatan mereka terhadap enzim α-amilase dan α-glucosidase. Supernatan daripada kultur *Lactobacillus acidophilus* mempamerkan aktiviti perencatan α-amilase yang paling ketara, dengan peratusan 79.01 ± 1.35%, manakala HKC *Lactococcus lactis* menunjukkan kadar perencatan 58.17 ± 3.08%. Untuk perencatan α-glucosidase, *Limosilactobacillus reuteri* menunjukkan kadar perencatan tertinggi dalam kedua-dua supernatan dan HKC, dengan peratusan masing-masing 68.08 ± 0.03% dan 67.78 ± 1.18%. Pecahan selanjutnya eksopolysaccharide (EPS), molekul kecil dan protein telah dijalankan, mendedahkan bahawa EPS yang diekstrak daripada supernatan *L. acidophilus* mempamerkan kesan perencatan yang paling luar biasa, dengan perencatan 95.90% dalam ujian α-amilase dan perencatan 75.51% dalam ujian α-glucosidase, mengatasi komponen pecahan lain. Kajian secara sistematik menyiasat pelbagai faktor parameter penulenan dalam ATPS, termasuk kepekatan etanol, jenis dan kepekatan cecair ionik, jenis dan kepekatan garam dan pH garam. Kaedah ini juga menapis kandungan protein dan determinasi purifikasi pekali sesekat. Kandungan EPS yang telah disucikan daripada *L. acidophilus* (63.30µg/mL) dan *L. reuteri* (146.48µg/mL) diperolehi dalam keadaan optimum ATPS yang terdiri daripada 30% (w/w) etanol, 25% (w/w) dipotassium hidrogen fosfat pada pH 10 dan 2% (w/w) 1-butil-3-methylimidazolium oktil sulfat. Kandungan EPS yang diekstrak ditentukan menggunakan kaedah asid sulfurik fenol dan dibandingkan dengan ekstrak mentah. Dalam ujian perencatan α-amilase, kadar perencatan didapati 92.52% *(L. reuteri)* dan 90.64% *(L. acidophilus)*, manakala dalam ujian perencatan α-glucosidase, kadar perencatan ialah 73.58% *(L. reuteri)* dan 68.77% *(L. acidophilus),* berdasarkan parameter dioptimumkan yang dipilih dalam ATPS. Keputusan ini menunjukkan bahawa EPS yang telah dipurifikasikan berasal daripada postbiotik *Lactobacillus spp.* yang berpeluang sebagai agen antidiabetik yang berpotensi

**PRODUCTION OF POSTBIOTIC BY LACTIC ACID BACTERIA THROUGH FERMENTATION PROCESS AS POTENTIAL ANTIDIABETIC AGENT**

## ABSTRACT

In response to the notable adverse health effects associated with the use of chemically synthesized antidiabetic drugs for individuals with diabetes, a comprehensive investigation was undertaken to explore the potential of postbiotic compounds produced by lactic acid bacteria (LAB) as novel antidiabetic agents, with a particular focus on their inhibitory effects compared to acarbose. Seven LAB strains were cultured to obtain supernatants and heat-killed cell (HKC) which was exposed to 80°C, which were subsequently evaluated for their inhibitory activities against α-amylase and α-glucosidase enzymes. The supernatant from *Lactobacillus acidophilus* TP7 culture exhibited the most significant α-amylase inhibitory activity, with a percentage of 79.01 ± 1.35%, while HKC of *Lactococcus lactis* C22 displayed an inhibition percentage of 58.17 ± 3.08%. For α-glucosidase inhibition, *Limosilactobacillus reuteri* BM5 showed the highest inhibition percentage in both the supernatant and HKC, with percentages of 68.08 ± 0.03% and 67.78 ± 1.18%, respectively. Further fractionation of exopolysaccharide (EPS), small molecules, and proteins was carried out, revealing that the crude EPS extracted from *L. acidophilus'*s supernatant exhibited the most remarkable inhibitory effects, with 95.90% inhibition in the α-amylase test and 75.51% inhibition in the α-glucosidase test, surpassing the other fractionated components. The study investigated various purification factor parameters in Aqueous Two-Phase System (ATPS) to purify EPS, such as ethanol concentration, type and concentration of ionic liquid, type and concentration of salt and pH of salt. This method was also able to remove protein and determination of purification by partition coefficient. Purified EPS contents from *L. acidophilus* (63.30µg/mL) and *L. reuteri* (146.48µg/mL) were obtained under optimum conditions of ATPS which consisted of 30% (w/w) ethanol, 25% (w/w) dipotassium hydrogen phosphate at pH 10 and 2% (w/w) 1-butyl-3-methylimidazolium octyl sulfate. The extracted EPS content was determined using phenol sulphuric acid method and compared with crude extract. In α-amylase inhibition tests, the inhibitory percentage was found to be 92.52% (*L. reuteri*) and 90.64% (*L. acidophilus*), while in α-glucosidase inhibition tests, the inhibitory percentage was 73.58% (*L. reuteri*) and 68.77% (*L. acidophilus*), based on the optimized parameters selected in ATPS. These results suggest that the purified EPS derived from the postbiotics of *Lactobacillus* spp. hold promise as potential antidiabetic agents.

# CHAPTER 1

# INTRODUCTION

## 1.1 Diabetes

Diabetes mellitus prevalence has risen compared to 108 million during 1980 towards 422 million within 2014, according to the data. Global prevalence increased to 8.5% in 2014 from 4.7% in 1980. In 2012, 2.2 million passings were accounted for before age of 70 because of hyperglycemia in blood in basically the same manner in 2016, roughly, 1.6 million passings were brought about by diabetes (Xiao *et al*., 2019). But that prevalence was estimated a decades ago. In recent record, it was stunning with the increasing percentage of this leading disease. Some nations witness a decline in diabetes incidence. However, diabetes prevalence has mount. It rises in most other nations. These are either developed or developing countries. This has occurred over recent decades. According to the IDF, 9.3% of global adult population had diabetes in 2019. This equated to 463 million individuals. However, in 2030 the number is expected to rise. It will increase to 10.2% or 578 million individuals. By 2045 an increased figure of 10.9% or 700 million individuals. This will be the case if no prevention methods are implemented. In 2017 nearly half the total diabetic population (50.1%) were undiagnosed. This represents around 374 million people. These are individuals aged 18-99 years. Along similar lines prediabetes is a problem too. It is estimated to affect 374 million people or 7.5% of global population in 2019. In 2030 the number is predicted to rise to 454 million or 8.0%. By 2045, the projection goes to 548 million. That equates to 8.6%. With prediabetes the majority are under 50 years the percentage is 48.1. Type-2 diabetes is another major concern in this category. It reduces average lifespan by a decade or so (Saeedi *et al*., 2019).

Malaysia has the highest incidence of diabetes in the Western Pacific region, alongside some countries in the world. The cost burden adds up to about 600 million US dollars annually. The diabetes rate increased from 11.2% in 2011 to 18.3% in 2019, which is an increase of 68.3%. As per a national survey in 2019 of Malaysia, an estimated 3.7 million adults aged 18 years and above were diabetic with 49% (3.6 million) of the cases undiagnosed. An increase in cases is foreseen which states that by 2025 an estimated 7 million Malaysian adults aged 18 and above will be affected, which would make a serious public health threat with a diabetes prevalence rate of 31.3%. The reports from Malaysia indicate relatively wide range of diabetes prevalence rate of 7.3% to 23.8%. All these statistics denote that the projection of increasing prevalence of diabetes is attributed to a multitude of factors such as population growth, aging populations, urbanization, higher obesity levels and low physical activity (Akhtar *et al*., 2022).

## 1.2 Challenges with modern medicine

The majority of these pharmacological medication classifications have detrimental side effects. For example, sulfonylurea induces hypoglycemia, which, although typically mild to severe, can result in weight gain, gastrointestinal issues, mortality from cardiovascular disease, a slight headache, and deadly complications. Temporary nausea, anorexia or diarrhea, abdominal pain, lactic acidosis with multiple renal impairment, and renal hypoperfusion can all be side effects of the drug metformin, which belongs to the biguanide class. The need for a novel class of medicinal anti-diabetic chemicals to combat diabetes-related issues, including numerous secondary consequences, is fueled by these circumstances.

However, the affordability for the insulin medicine for diabetes is quite challenging due to its price and also inappropriate usage of the medicine. Poverty is related with low health vitality; hence inappropriate usage of the drug will make it worst and discrepancy. An approximate quoted price to supply diabetes medicine cost roughly $12.5 billion for developing countries (Wirtz *et al.*, 2017). Based on the World Health Organization (WHO) report, around 70-80% of the total populace depend on non-conventional medication, mostly from natural sources. In developing nations, its demand is gradually rising. In Malaysia, the prevalence of type 2 diabetes (T2D) among adults is approximately 1 in 6, with annual expenditures for disease management estimated at RM 2.04 billion. This financial burden is projected to increase over the next ten years, largely due to the aging demographic. Cost related to diabetic drugs varies by average from RM275.83 to RM6124.69 annually (Sim *et al.*, 2023).

### 1.3 Essential Gut Microbiota

The human gut microbiome consists of trillions of microbes, each of which profoundly impacts one’s gut health, immune response, and even mental health. As metabolic disorders are linked with the imbalance of gut microbiota, it is critical to take care of one’s gut. Prebiotics, probiotics, synbiotics, and postbiotics (PPSP) are all part of functional food ingredients that have potential in improving gut health. Prebiotics are non-digestible carbs that are broken down in the large intestine by good gut bacteria, whereas probiotics are live microrganisms that provide a plethora of health benefits when consumed in sufficient amounts. When probiotics and prebiotics are combined together, they form synbiotics. Postbiotics are molecules that provide health benefits such as active compounds made during the fermentation of probiotics. According to new research, functional food ingredients such as PPSPs have the ability to inhibit the creation of metabolites, enhance the strength of the intestinal barrier, and shift the gut flora to manage metabolic diseases (Al-Habsi *et al*., 2024). The differences between Prebiotics, Probiotics, Synbiotics and Postbiotics summarized in Table 1 below (Mao *et* al., 2023):

**Table 1** Shows the difference between Prebiotics, Probiotics, Synbiotics and Postbiotics

| Classification | Prebiotics | Probiotics | Synbiotics | Postbiotics |
|---|---|---|---|---|
| Annotation | A substrate that is specifically used by host microorganisms to provide a health advantage. | Live microorganisms which, when provided in sufficient quantities, offer a health advantage to the host. | A composition that includes viable microorganisms along with substrates that are preferentially utilized by the host's microorganisms, which provides a health advantage to the host. | The formulation of non-living microorganisms and/or their constituents that provides a health advantage to the host. |
| Group | Polyunsaturated fatty acids and compounded linoleic acids; oligosaccharides; oligosaccharides in | *Bifidobacterium* species include *adolescentis, animalis, bifidum, breve,* and *longum,* while *Lactobacillus* species encompass *acidophilus,* | Complementary refers to the combination of prebiotics and probiotics, while synergistic denotes the interaction between live microorganisms and their substrates. | Inactivated strains, including *Bacteroides xylanisolvens, Apilactobacillus kunkeei,* and *Saccharomyces boulardii;* bacterial lysates; and formulations containing Spirulina. |

| | | | | |
|---|---|---|---|---|
| | human milk; phytochemicals and phenols; fermentable quickly | *casei, fermentum, gasseri, johnsonii, paracasei, plantarum, rhamnosus,* and *salivarius.* | | |
| Benefits for health | Metabolic well-being; Feelings of fullness; Enhanced uptake of calcium and various minerals, skeletal health; Dermatological health; Gastrointestinal | The proper formation of a healthy digestive system is essential for addressing conditions such as infectious diarrhea, antibiotic-associated diarrhea, and ulcerative colitis. Additionally, a robust immune system is | Management strategies for non-alcoholic fatty liver disease (NAFLD), obesity, metabolic syndrome, type 2 diabetes mellitus (T2DM), glycemic control, irritable bowel syndrome (IBS), chronic kidney disease (CKD), dyslipidemia, polycystic ovary syndrome (PCOS), | Novel antimicrobials; Agents designed for targeted anti-inflammatory and immunoregulatory effects, as well as those that improve the efficacy of vaccinations; New signalling molecules influencing gut-related pain, sensation, secretion, and motility; Fermented formulas for infants and bacterial lysates. |

| | | | | |
|---|---|---|---|---|
| | health; Allergic responses; Bowel regularity; Immune system function in older adults. | crucial for preventing allergic diseases, reducing inflammation, and improving the body's ability to combat infections. | Alzheimer's disease (AD), and inflammatory conditions; prevention of surgical infections and complications, including sepsis in neonates, as well as Alzheimer's disease; and the eradication of *Helicobacter pylori*. | |
| Mechanism | Regulation of short-chain fatty acid (SCFA) synthesis; Support of advantageous microbial | Resistance to colonization; Restoration of disrupted microbiota; Production of short-chain fatty acids (SCFAs); Enhanced turnover of intestinal | The complementary approach integrates prebiotics, which specifically target indigenous beneficial microorganisms, alongside probiotics. In contrast, the synergistic approach involves | Alteration of indigenous microbiota, immune system reactions, and overall metabolic processes; Improvement of epithelial barrier integrity; Control of systemic communication through the nervous |

| | | | | |
|---|---|---|---|---|
| | communities; Metabolism of bile salts; Modification of bacterial proliferation and their interaction with the immune system; Increased release of satiety hormones such as peptide YY and GLP-1; Modulation of immune responses. | epithelial cells; Modulation of gut transit; Competitive inhibition of pathogens; Synthesis of vitamins; Metabolism of bile salts; Strengthening of the intestinal barrier. | the selection of substrates that are utilized by the concurrently administered live microorganisms, thereby augmenting their functional efficacy. | system. |

## 1.4 Microorganism intervention against diabetes

A high fascination towards gut microbiota is popular among scientific researcher in this modern era. Hence, the utilization of natural, safe, and financial choices like the utilization of probiotics/postbiotics can be used to adapt to these issues and also due to concerns about supplementing with live probiotic microorganisms and the numerous benefits of postbiotics.

Despite the beneficial effects of postbiotics as an antidiabetic agent which was obtained from probiotic derivatives, probiotics are food-grade microorganisms that when taken in sufficient quantities it will provide health benefits without affecting human health. In such manner, the utilization of metabolites derived LAB, which is referred to the present time as "postbiotic", is recommended as a reasonable elective methodology as potential antidiabetic agent. Treatments to control diabetes are currently causing users more concern in modern medicine.

## 1.5 Postbiotics and its potential

Postbiotic address to byproducts or derivatives from probiotics namely by food grade microorganisms which possessed human wellbeing benefits when taken in decent/advised amounts. Postbiotic compromises the cell structure and by-products such as metabolites/component which are secreted by the respective microbes. Commonly can be obtained by isolating the by-product which undergoes cell lysis or by centrifugal method. Even though most of the postbiotics were obtained from bacteria (*Lactobacillus* and *Bifidobacterium* more often), there were also some investigations initiated for fungal origin postbiotic. Based on the recent postbiotic definition published in International Scientific Association for Probiotics and Prebiotics (ISAPP), it also compromised inanimate microorganisms. In the current approach, there were also postbiotic available commercially in the form of supplements and introduced in foods as well. The intention of the incorporation is for their benefit in use for alimentary tract and digestive tract or immune linked diagnosis. Based on retrospective scrutiny and findings, it suggested that prospect of postbiotic is huge and opens future application in clinical approach mainly in diabetes mellitus. The wide prospect of postbiotic due to its multiple benefits and advantages possessed. Postbiotics are safe in nature as they do not bring any virulent/pathogenic factor or harmful substances which will trigger the immunization process. They also reduced the adverse effects in at risked individuals such as pregnant women, premature infants, elderly adults and immunoreacted individuals. Besides that, postbiotics also have a significant characteristic where it doesn’t have a risk of transmitting antibiotic defiant genes to infective or mutualistic bacteria. They have extended durability and improved shelf life, which is an important factor during administrative to human consumption. The major benefit of postbiotic is the ability to produce postbiotic in large scale. Due to the modern growth of the world and population, most of the industries have space constraints where they are unable to produce more desired products. Examples are plant-based derivatives, antidiabetic agent, antibiotic and so on, which need huge space to cultivate the desired plants for further studies/investigation. However, there is no such constraint by using bioreactor to produce postbiotic producing bacteria in large scale. This also reduced the space consumption, promoted large scale production, enabled to control parameters to have a significant growth and yield of postbiotic/byproduct of interest. Thus, the studies towards bacteria using bioreactors gained popularity compared to typical plant growth and cultivation method of studies. Interestingly, postbiotic also can be used concurrently administration with antidiabetic and antifungal agents (Cabello-Olmo *et al*., 2021).

Metabolites derived from microbes are essential for classification aid. Besides metabolites, molecular shapes also play a crucial role in the classification. Below is the summarized Table 2 on postbiotic classification based on the bacteria component.

**Table 2** Classification of postbiotic based on its component and example of its derivatives.

| **Postbiotic components** | **Derivatives** |
|---|---|
| Cell free extracts and lysates | Culture supernatants, inanimate microorganism, biosurfactants (Cabello-Olmo *et al*., 2021) |
| Microbial derived metabolites | Enzymes, proteins, EPS, peptides, organic acids, vitamins, antimicrobial peptides, minerals, bacteriocins, extracellular vesicles (Wang *et al*., 2021) |
| Outer structure/shape derivatives | Cell wall components, cell surface fractions, polymers teichoic acids, peptidoglycan derived muropeptides, lipoteichoic acid, wall teichoic acid, peptidoglycans, pili type forms (Wang *et al*., 2021) |

There are plenty of methods and techniques to extract and purify the postbiotic compounds. The principle is to disrupt the cellular wall/cellular membrane/extracellular layer in order to release the intracellular component which is classified as postbiotic compounds. Method or techniques commonly used is ironically solvent extraction, deproteinization and precipitation (eg. ethanol precipitation, ammonium sulphate precipitation), hydrophobic interaction chromatography electrophoresis separation and evaluation using liquid chromatography or sonification. In order to kill the bacteria, several methods/techniques can be used which is exerting high temperature, high pressure, irradiation and sonification.

The reasoning for a broad utilization of LAB is to create high life span of usability food, to upgrade its safety level, flavor, appearance and surface as well as to work on its physiological and sanitary worth because of the presence of viable cells and important metabolites obtained from LAB (Castellone *et al*., 2021).

The LAB present in food enhance the digestibility of proteins, moderate the release of fatty acids, and support human health through inhabiting the gastrointestinal tract. These desirable properties of LAB are attributed, in part, to their metabolic processes involving enzymes such as lipases, proteases, and antibacterial proteins. The LAB strains presenting higher enzymatic activities may offer improved functionality for applications in foods (García-Cano *et al*., 2019). For the manufacturing and preservation of food, as well as for imposition of positive effects on human well-being, all of these characteristics exhibit fascinating technological attributes.

In a normal natural carbohydrate metabolic mechanism, the carbohydrate will be digested by hydrolyzing enzyme known as α-amylase and followed by α-glucosidase to glucose in intestinal tract. The glucose will be absorbed into bloodstream and excessive glucose will cause insulin to be released by the pancreas. This action will cause liver to take glucose out of the bloodstream and store it as glycogen. Contradictory, when there is low blood glucose level, it will cause pancreas to produce glucagon which will stimulate liver to release glucose into the bloodstream. That's the layman's overview on how the carbohydrate breakdown process occurs. In T2D, the insulin will not be secreted enough to reduce the glucose in bloodstream and in some cases, pancreas unable to secrete insulin at all. This condition will increase the saturation and level of glucose in bloodstream. In order to curb this issue, a systemic approach was figured out to inhibit the α-amylase and α-glucosidase enzyme from preventing carbohydrate breakdown into glucose in postprandial hyperglycemia. There were plenty of potential inhibitors was experimented chemically and also synthetically. The inhibitors will attach to the enzyme's docking or active site and prevent the breakdown of carbohydrate to glucose (Cabello-Olmo *et al*., 2021).

## 1.6 Problem Statement

The affordability of the insulin medicine for diabetes is quite challenging due to its price and also inappropriate usage of the medicine. Even though, price for pharmaceuticals can be subject to change frequently due to various factors such as manufacturing adjustments, government regulations, and currency fluctuations. Poverty is related to low health vitality; hence inappropriate usage of the drug will make it worse and discrepancy. However, chemical and synthetic inhibitors do impose some side effects to the gastrointestinal tract. Commonly known sides effects are diarrhea, stomach pain and bloating. α-Amylase and α-glucosidase inhibitor derived from plant and bacteria's compound is lesser in the term of side effect. EPS was less researched to curb this issue, limited understanding and due to its multiple binding side/structure made it difficult to predict the effectiveness of this compound as an alternative for insulin. The production and purification of EPS are challenging and expensive, besides lack of standardization. Thus, this study is conducted to select the LAB which has the highest inhibition percentage of α-amylase and α-glucosidase activity and to identify the postbiotic compound produced by the selected LAB which has potential to be validated as antidiabetic agent.

## 1.7 Objective

1. To evaluate the *in-vitro* efficacy of postbiotics of LAB as anti-diabetic agent as compared to standard anti-diabetic drugs such as acarbose.
2. To investigate the optimum purification parameters in aqueous two-phase system for improvement of the recovery and yield of EPS extracted from LAB.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Introduction

The advantageous characteristics of probiotics have consistently attracted scholarly attention. Probiotics are essential for the maintenance of the Gastrointestinal Tract (GIT) health, which in turn is crucial for regulating various bodily functions. The efficacy of probiotics can be significantly improved when they are combined with prebiotics, resulting in a formulation known as synbiotics. This combination not only enhances the viability and stability of probiotic organisms but also suppresses the proliferation of harmful microbial strains. *Lactobacillus* and *Bifidobacterium* species are the most frequently utilized probiotics. Other microbial species that may serve as probiotics include *Bacillus, Streptococcus, Enterococcus,* and *Saccharomyces*. Probiotics have potential applications in the management of conditions such as diabetes, obesity, inflammatory diseases, cardiovascular issues, respiratory ailments, central nervous system disorders, and various digestive problems (Sarita *et al*., 2024). Evidence that the resident gastrointestinal microbial (GM) is one of the environmental aspects that support growth and development of DM has grown in recent years (Cabello-Olmo *et al*., 2019).

Both T1D and T2D (Gurung *et al*., 2020) have been reported to have a leaky gut, and a change in intestinal permeability has been connected to DM. In view of this, numerous studies and research have highlighted the therapeutic potential of dietary interventions that modulate the microbiota in various forms of diabetes mellitus. Postbiotics are emerging as potential agents for DM prevention or management, despite the fact that probiotics, prebiotics (Hansen *et al*., 2019) and fermented foods (Cabello-Olmo *et al*., 2019) have served as a reference for microbiome-based interventions. Great proof for this can be found in exploratory examinations in various models of diabetes mellitus and narrowly any human preliminaries (Barros *et al*., 2021).

Over the past two decades, Lactobacillus has become the leading term for probiotics, achieving significant recognition in scientific discussions and studies. Consequently, it is essential to consider the probiotic capabilities of LAB genera carefully and evaluate each one individually to assess their appropriateness as probiotics. Several LAB genera are considered probiotics, though there is disagreement among scientists. The probiotic LAB strains that have been studied the most are *Lactobacillus acidophilus* NCFM, *Lactobacillus acidophilus* LA-5, *Lactobacillus casei* DN-114 001, *Lacticaseibacillus casei* strain Shirota, *Lacticaseibacillus casei* Zhang, and *Lactobacillus rhamnosus* GG (ATCC531013). (Ağagündüz *et al*., 2021).

One of the major factors that plays part in the development of diabetes is impaired glucose metabolism and insulin resistance. Experiments have evidenced that LAB can influence glucose metabolism by improving insulin secretion and sensitivity. LAB stimulate the production of insulin, a hormone responsible for regulating blood glucose levels. This increased insulin secretion helps to normalize elevated blood glucose levels in individuals with diabetes. Furthermore, LAB have been shown to enhance insulin sensitivity, enabling cells to better utilize glucose for energy. By improving insulin sensitivity, LAB can help to overcome insulin resistance and promote better glycemic control (Cabello-Olmo *et al*., 2021).

Numerous research efforts indicate that LAB have various physiological roles, such as fortifying the intestinal mucosa, boosting immune regulatory functions, enhancing the body's antioxidant capabilities, lowering cholesterol levels, and combating biofilm formation (Hakim *et al*., 2023). Moreover, research has shown that these characteristics of LAB are strongly linked to the substances generated by their metabolic processes, including bacteriocins and exopolysaccharides (EPSs). Among these, EPSs have garnered significant interest because of their distinctive characteristics and roles, making them a focal point of research (Liang *et al*., 2024).

*Lactobacillus reuteri (L. reuteri)* is a well-studied probiotic bacterium that colonizes a wide array of mammalian species. In humans, *L. reuteri* can be found in various anatomical sites, including the gastrointestinal tract, urinary tract, skin, and breast milk. The occurrence of *L. reuteri* varies among individuals. This bacterium plays a role in preventing certain ailments, as children who are administered *L. reuteri* while healthy exhibit a reduced likelihood of developing diarrhea. Comparative research suggests that *L. reuteri* is more effective than other probiotics in providing protection against intestinal infections. High doses of the chemotherapy agent methotrexate can lead to severe enterocolitis; however, *L. reuteri* has been shown to significantly alleviate the symptoms and signs of methotrexate-induced enterocolitis in rat models, including the issue of bacterial translocation. Furthermore, *L. reuteri* has been recognized for its potential to improve dental health by effectively targeting and eliminating *Streptococcus* mutans, a bacterium linked to dental caries. Among the probiotics studied, *L. reuteri* was the sole strain that demonstrated the capacity to inhibit Streptococcus mutans. Additional research confirmed that *L. reuteri* does not adversely affect dental health prior to human trials. Subsequent clinical studies indicated that individuals whose diets included *L. reuteri* exhibited a markedly lower presence of *Streptococcus* mutans in their oral microbiome (Shah *et al*., 2024).

*Lactobacillus reuteri* is associated with numerous health benefits, including the prevention and management of urogenital conditions and bacterial vaginosis in women, as well as atopic disorders, food allergies, and the reduction of dental caries. Additionally, research has explored its potential to prevent colitis and reduce interactions between P-selectin-associated leukocytes and platelets with endothelial cells, highlighting its relevance in intestinal diseases. Extensive studies have also been conducted on its ability to inhibit the proliferation of pathogenic bacteria, yeasts, and other microorganisms, thereby underscoring its promise as a beneficial probiotic for addressing gastrointestinal and urogenital issues, including infant colic (Sarita *et al*., 2024).

*Lactobacillus acidophilus* is a Gram-positive, rod-shaped bacterium that possesses several important attributes. This microorganism plays a significant role in human health, contributing to the management of various conditions, including lactose intolerance, nonalcoholic fatty liver disease, irritable bowel syndrome, and hypercholesterolemia, while also providing protection against *Helicobacter pylori* infections. Predominantly found in the gastrointestinal tract, it is essential for maintaining overall health. Specifically, *L. acidophilus* contributes to the balance of the microbiota, aids in digestion, improves nutrient absorption, and strengthens the immune response. Furthermore, studies indicate that this bacterium may lower serum cholesterol levels, stabilize gut microbiota, and exhibit anti-cancer properties. *L. acidophilus* also has the ability to inhibit the growth of harmful bacteria in the intestines, regulate the composition of intestinal flora, and enhance immune resistance, thereby promoting growth and improving overall immunity. Various factors, including salinity, temperature, carbon sources, and nutrient availability, can influence the growth of *L. acidophilus*. Research has indicated that fluctuations in salinity and temperature during fermentation can affect the survival and proliferation of this bacterium. The availability of essential nutrients, such as amino acids, riboflavin, and minerals like manganese (Mn2+), is also critical for enhancing the growth of *L. acidophilus* in fermented products. Additionally, optimizing the carbon source and its concentration in the fermentation medium is crucial for promoting the growth, phenolic production, and antioxidant activity of *L. acidophilus*. Understanding and managing these conditions is essential for maximizing the growth and bioactive potential of *L. acidophilus* throughout various fermentation processes (Liu *et al*., 2024).

*Lactobacillus acidophilus* is associated with numerous health advantages. Studies have demonstrated that *L. acidophilus* is instrumental in promoting cardiovascular health, alleviating lactose intolerance, preventing cancer, regulating immune responses, and addressing gastrointestinal disorders. Furthermore, *L. acidophilus* possesses antimicrobial properties that combat a diverse array of pathogenic bacteria, thereby enhancing its protective functions. The immunomodulatory characteristics of *L. acidophilus* can trigger anti-inflammatory mechanisms, improve phagocytic activity, stimulate the production of defensins, and adjust intestinal permeability, all of which are vital for bolstering immunity and maintaining optimal gut health. Clinical investigations have revealed that the consumption of acidophilus milk fortified with *L. acidophilus* can be beneficial in managing ailments such as diarrhea, constipation, and Alzheimer's disease, indicating its potential as a complementary treatment for various health conditions (Liu *et al*., 2024).

The synthesis and utilization of EPS derived from *Lactobacillus acidophilus* and other LAB strains present considerable potential for improving food quality and enhancing human health due to their multifunctional characteristics. Extensive research has been conducted on the EPS production by *Lactobacillus acidophilus*, particularly the BCRC 10695 strain, highlighting its advantageous effects and prospective applications within the food and pharmaceutical sectors. The production methodology necessitates the optimization of the culture medium's composition, incorporating carbon sources like sucrose, nitrogen sources such as yeast extract, and surfactants including polysorbate 80. Through the application of response surface methodology (RSM), optimal concentrations were identified as 10.15 g/L sucrose, 25 g/L yeast extract, and 2 g/L polysorbate 80, culminating in an EPS yield of 923.7 mg/L following 96 hours of cultivation. *Lactobacillus acidophilus* is recognized for its ability to produce EPS, which confer various functional benefits, including improved taste and texture, alongside health advantages such as immunomodulatory and anticancer properties. Recent findings indicate that EPS remains effective in both normoxic and hypoxic environments, enhancing the expression of specific genes associated with cancer suppression. The optimization of EPS production was achieved through targeted design methodologies, resulting in a maximum EPS concentration of 597 mg/L during batch cultivation. Additionally, *Lactobacillus acidophilus* was isolated from rice bran sourdough, which yields a unique EPS characterized by high carbohydrate content and antioxidant capabilities. Various analytical techniques were employed to identify and characterize the EPS, confirming its structural components, including glucose, galactose, and maltose. The EPS produced by *Lactobacillus acidophilus* demonstrated notable antioxidant activity, indicating its potential as a valuable functional ingredient (Liu *et al*., 2024).

EPS is important in the food sector, being extensively utilized and showcasing various functional attributes. In addition to its emulsifying properties (Sharma *et al*., 2020). Consequently, EPS generated by LAB has shown remarkable promise in the pharmaceutical sector and is deemed to offer considerable advantages for human health. Through continued research, the practical use and medical importance of EPS generated by LAB will be more thoroughly investigated and applied.

## 2.2 Aqueous Two-Phase System

The Aqueous Two-Phase Systems (ATPS) appear to be a method that serves as a base for more than one functional possibility- separation, concentration, and purification. This all makes it perfectly possible that such a technology easily qualifies as an ideal extraction technology. An ATPS is an extraction-driven technology based on the combination of two polymers or a polymer with salt, alcohol with salt, or ionic liquid with salt, polymer, and carbohydrate, or even surfactants in water. A suitable selection of the phase forming components guarantees the formation of two distinct layers at equilibrium, each of which predominately contains one of the system components. When the idea of ATPS was developed, the key substance present in the fermentation broth was transferred to one phase, and the remaining material (primarily containing the impurities and contaminants) was moved to the other phase. The idea can effortlessly be adapted to extract the valuable into products (or, alternatively, extractors of the contaminants) from waste streams of various industries that would have otherwise been discard away (Varadavenkatesan *et al*., 2021). Even though ATPS have accomplished impressive efficiency and purity yields, there is still a significant gap between their bench-scale use and their possible industrial uses (Torres-Acosta *et al*., 2019).

## 2.3 Separation and Purification of Exopolysaccharides (EPS) Using Aqueous Two-Phase System (ATPS)

Exopolysaccharides (EPS) are large molecular-weight polysaccharides produced by microorganisms such as bacteria, fungi, and algae (Freitas *et al*., 2017). They have attracted considerable interest because of their extensive applications in industries such as food, pharmaceuticals, and biomedicine. EPS demonstrate a range of functional characteristics including improved viscosity, emulsifying properties, and antioxidant effects. Effective methods for separation and purification are crucial to realize their complete capabilities. Among these methods, the aqueous two-phase system (ATPS) has surfaced as an effective and environmentally friendly approach for EPS recovery.

Exopolysaccharides (EPS) are generated through microbial fermentation and typically exist within intricate mixtures that include cells, proteins, nucleic acids, and various impurities. Conventional methods for separation and purification, including solvent precipitation, membrane filtration, and chromatographic techniques, frequently encounter difficulties such as inadequate selectivity, elevated energy requirements, and reliance on hazardous solvents (Chen *et al*., 2018; Xu *et al*., 2019). These challenges have prompted researchers to investigate alternative approaches, such as aqueous two-phase systems (ATPS), which present benefits including biocompatibility, ease of use, and scalability.

ATPS represents a liquid-liquid extraction method characterized by the creation of two immiscible aqueous phases (Rosa *et al*., 2018; Silva *et al*., 2020). This system is generally established by combining two incompatible polymers, such as polyethylene glycol (PEG) and dextran, or by mixing a polymer with a salt, for instance, PEG and potassium phosphate, in an aqueous environment. The application of ATPS has been extensively recognized for its effectiveness in the separation and purification of various biomolecules, including proteins, nucleic acids, and polysaccharides.

Numerous studies have illustrated the effectiveness of ATPS in the separation and purification of EPS (Zhu *et al*., 2019; Li *et al*., 2021) as detailed below:

a) **Polyethylene Glycol (PEG)/Salt Systems:** PEG/sodium citrate and PEG/potassium phosphate systems have been documented to attain significant recovery and purity of EPS. These systems present benefits including reduced viscosity and swift phase separation.

b) **Polymer/Polymer Systems:** PEG/dextran systems have been employed for the mild separation of EPS, especially in scenarios where preserving the bioactivity of EPS is essential.

c) **Customized ATPS:** Researchers have investigated the application of innovative polymers and salts to improve the partitioning of particular EPS. The use of functionalized polymers and ionic liquids has demonstrated potential in increasing the efficiency of separation processes.

The method, however, possesses specific limitations that warrant attention. Achieving phase stability can be challenging, especially when managing intricate EPS mixtures or fluctuations in environmental conditions. Furthermore, the transition of the ATPS method to industrial applications may encounter obstacles related to the duration of phase separation, the expense of phase-forming components, and the necessity for system optimization. Nevertheless, current research endeavours are focused on overcoming these challenges through the development of advanced materials and innovative processes.

## 2.4 Research conducted by using postbiotic compounds against diabetic

Recent studies have investigated the efficacy of postbiotic compounds in the management of diabetes, focusing on their anti-inflammatory effects, ability to regulate metabolism, and capacity to modulate gut microbiota.