POLY-GAMMA-GLUTAMIC ACID: A COMPREHENSIVE OVERVIEW OF BIOSYNTHESIS, CHARACTERISTICS, AND EMERGING APPLICATIONS

Mohammed Jabbar Hafiz Al-wahili, Alaa Jabbar Abd Al-Manhel*, Rawdah Mahmood Al-Ali, Ammar B.Altemimi*

Address(es):
Department of Food Science, College of Agriculture, University of Basrah, Basrah 61004, Iraq.

*Corresponding author: alaa.abd@uobasrah.edu.iq

ABSTRACT

Poly-gamma-glutamic acid (PGA) is a naturally occurring biopolymer that has been receiving increasing attention in various fields, including the food, medical, and cosmetic industries. This review provides a comprehensive overview of PGA, discussing its biosynthesis, characteristics, and emerging applications. The biosynthesis of PGA is discussed in detail, highlighting the various microorganisms that are capable of producing PGA and the factors that affect its production. The structure and molecular weight of PGA are also described, as well as its solubility and biodegradability. These properties make PGA a highly versatile material that can be utilized in a wide range of applications. The review also provides an in-depth analysis of the emerging applications of PGA. In the food industry, PGA has been used as a natural preservative, a thickener, and a flavor enhancer. In the medical field, PGA has shown promising results as a drug delivery system and a scaffold for tissue engineering. The challenges and future directions for the production and utilization of PGA are also discussed. This review article covers key aspects, including production process optimization, property improvement, and exploration of novel applications for PGA. It serves as a valuable resource for researchers and industry professionals who wish to explore the potential of PGA in diverse applications. The exceptional properties of PGA make it highly appealing, with a broad spectrum of potential uses across multiple industries.

Keywords: γ-PGA, biosynthesis, glutamic, Industrial applications, regulation

INTRODUCTION

Poly-gamma-glutamic acid (γ-PGA) is a type of biopolymer that is made up of monomers of D- and/or L-glutamic acid linked together by peptide bonds. It is typically found in nature and has several desirable properties, including water solubility, edibility, biodegradability, and a lack of immunogenicity (**Li *et al.*, 2022**). γ-PGA is produced by certain strains of Bacillus bacteria through a fermentation process. This homogenous polyamide is both edible and environmentally safe for humans to use. The γ-PGA molecules are made up of amide linkages between the gamma carboxyl and alpha amino groups, resulting in a unique chemical structure (**Mitsunaga *et al.*, 2016; Bajaj and Singhal, 2011**). The process of microbial synthesis is a highly efficient and cost-effective method for producing poly-gamma-glutamic acid (γ-PGA) (**Tamang *et al.*, 2016**).

Producers of γ-PGA can be found among bacteria, archaea, and eukaryotes (**Kumar *et al.*, 2023**). Once the bacteria have produced the γ-PGA, the biopolymer must be extracted from the submerged culture (**Azarhava *et al.*, 2020**). This extraction process typically involves four main steps. First, the culture is centrifuged to create a cell-free supernatant. Next, heavy-weight γ-PGA is precipitated using an alcohol such as ethanol, at a ratio of one to three volumes. After this, the crude γ-PGA solution is dialyzed against a dialysis tube to remove low molecular weight impurities. Finally, deproteinization and ion-exchange chromatography are utilized to purify the PGA even further (**Azarhava *et al.*, 2020**).

The γ-PGA has a wide range of applications in various fields, including food processing (**Guo *et al.*, 2023**), Agriculture (**Shi *et al.*, 2023**), medicine (**Zhang *et al.*, 2023**), cosmetics (**Liu *et al.*, 2023**), and environmental protection (**Peng *et al.*, 2020**). The food industry has utilized γ-PGA as a food additive and preservative owing to its ability to improve food texture and stability (**Hu *et al.*, 2021**), and its antimicrobial properties (**Lee *et al.*, 2019**). Moreover, it has been demonstrated to enhance the solubility of specific food ingredients and to enhance the quality of meat products (**Lim *et al.*, 2023**). In agriculture, γ-PGA has been applied as a plant growth promoter due to its ability to enhance seed germination, increase plant growth, and improve stress tolerance in crops (**Mi *et al.*, 2022; Zhang *et al.*, 2017**). The potential applications of γ-PGA in the medical field include drug delivery, wound healing, and tissue engineering. Its biodegradability, biocompatibility, and capability to encapsulate drugs make it a promising delivery vehicle for therapeutic agents (**Balogun-Agbaje *et al.*, 2021; Hsieh *et al.*, 2005**). Due to its ability to enhance skin elasticity and retain moisture, γ-PGA has been employed as a moisturizing agent, skin conditioner, and anti-aging ingredient in cosmetics (**Chen *et al.*, 2020**). The γ-PGA has also been investigated for its potential to eliminate heavy metals and pollutants from wastewater due to its chelating properties and biodegradability, which makes it an ideal candidate for environmental protection purposes (**Liu *et al.*, 2022**). the diverse applications of γ-PGA make it a promising biopolymer with significant potential for further research and development in various fields.

The aim of this review was to offer a thorough summary of the biosynthesis and features of γ-PGA, along with investigating its potential applications in medical, food, and environmental industries. Furthermore, the review aims to examine the present challenges and future possibilities for utilizing γ-PGA in various domains. It provides an updated analysis of current research and recent advancements in γ-PGA, identifies knowledge gaps, and proposes areas for future investigation.

REVIEW METHODOLOGY

The researchers performed an extensive literature search using two databases, namely Web of Science and Scopus, which are based in London, UK, to gather information. They used the search terms "Poly-gamma-glutamic acid (γ-PGA)" and narrowed the search to the subtopic of "Food," as outlined in Figure 1. Web of Science was preferred due to its collection of articles, indexed journals, and user-friendly interface. The initial search for "Poly-gamma-glutamic acid (γ-PGA); biosynthesis, glutamic " yielded 2064 articles, with 1324 articles published since 2013. By including the keyword "food, agriculture" the search resulted in 423 articles published between 2000 and 2023, with 175 articles published since 2000. The figures shown in Figure 1 indicate a considerable rise in the application of γ-PGA in the food sector over the last 25 years. There is a noticeable increase in attention towards the Poly-gamma-glutamic acid (γ-PGA) aspect from 2000 to 2023, and a much more prominent growth in publications (nearly five per year) from 2015 to 2023.
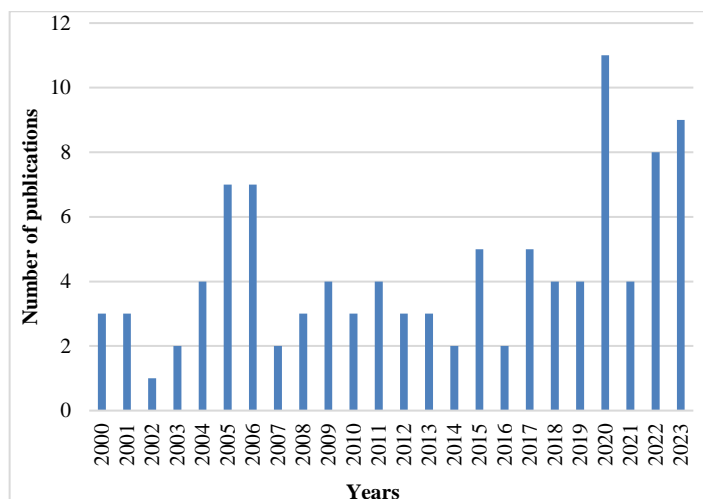
Figure 1 Comparison of the Number of Research Papers Published Over Time in Scopus and Web of Science Databases, with a Focus on Keywords Related to γ-PGA Biosynthesis, Glutamic Acid, Industrial Applications, and Regulation.

A BRIEF OVERVIEW OF γ-PGA

An analysis of the structural properties of γ-PGA

PGA is an uncommon and biodegradable homopolyamide composed of D and L glutamic acid units, which are both non-immunogenic and anionic. There are two types of PGA, α-PGA and γ-PGA, distinguished by the carboxy group's attachment (Fig. 2). The chemical synthesis of α-PGA involves nucleophile-initiated polymerization of L-glutamic acid's γ-protected N-carboxyanhydride. Microbial production of α-PGA is challenging, and the polymer can only be generated using recombinant technology, as per **Buescher and Margaritis (2007)**. The γ-PGA has the ability to adopt five distinct conformations, namely α-helix, β-sheet, helix-to-random coil transition, random coil, and enveloped aggregate. Variations in environmental conditions such as pH levels, concentration of polymers, and ionic strength can impact and alter the conformations of γ-PGA (**Ho *et al.*, 2006**). γ-PGA can exhibit different structures depending on the pH, with a mostly α-helical structure at pH 7 and a mainly β-sheet-based structure at higher pH. Additionally, the enantiomeric composition of γ-PGA can be modified through the extraction process post-fermentation. For example, γ-PGA that only contains l or d enantiomers dissolves in ethanol, while γ-PGA that has equal amounts of l and d enantiomers precipitates in ethanol. Therefore, modifying the enantiomeric composition of γ-PGA can be done to alter its properties (**Shih *et al.*, 2003; Bhat *et al.*, 2013**).
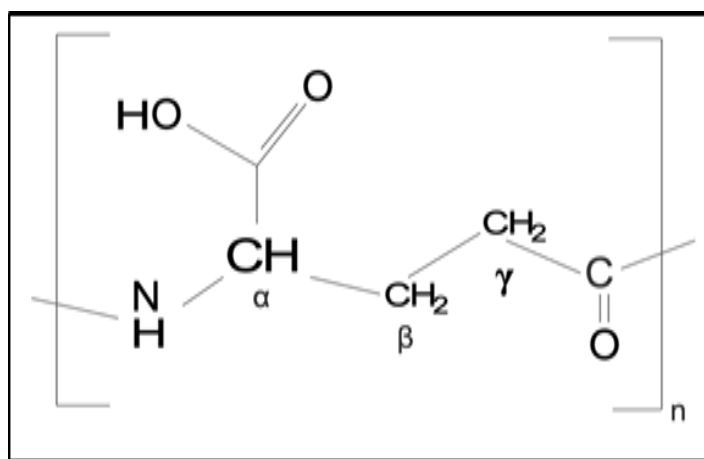

Figure 2 Structure of γ-PGA

The γ-PGA is a type of anionic polymer that can dissolve in water. However, its free acid form (H$^+$) cannot dissolve in water. On the other hand, the salt forms of γ-PGA, such as K$^+$, Na$^+$, NH$_4^+$, Ca^{2+}, and Mg^{2+}, can completely dissolve in water. Since, it is biodegradable and poses no harm to humans, γ-PGA can be used as a biological adhesive. It can also act as a natural moisturizer, with hygroscopic and moisturizing effects that are similar to those of Hyaluronic acid (HA). Due to its considerable adsorption abilities, γ-PGA is utilized as an absorbent substance. One of the most valuable types of Super Absorbent Polymers (SAPs) is Cross-linked γ-PGA (**Bae *et al.*, 2021; Najar and Das 2015**). The molecular weight of γ-PGA can vary from 100 KDa to 2,500 KDa (Kilo Daltons) and is affected by different factors, one of which is the duration of fermentation. Longer fermentation periods result in lower γ-PGA molecular weight due to the production of an enzyme that

causes the hydrolytic degradation of γ-PGA (**King *et al.*, 2000; Najar and Das 2015**). The high viscosity, unmanageable rheology, and complex modification of γ-PGA due to its molecular weight can restrict its industrial use. Thus, using polymers with varying molecular weights for different purposes and regulating molecular weight is critical for commercial advancements. Recently, different techniques have been employed to alter the molecular weight of γ-PGA, including changes in medium composition, alkaline hydrolysis, ultrasonic degradation, and microbial or enzymatic degradation (**Shih *et al.*, 2001**).

Production of γ-PGA via biosynthesis

Initially, γ-PGA is synthesized within the cell and later released into the fermentation broth (**Wang *et al.*, 2017**). L-Glutamic acid is used both as an inducer and as the source of the material for the production of γ-PGA. During the biosynthesis of γ-PGA, L-Glutamic acid is converted into its D-enantiomer through racemization, and then both the L- and D-forms are combined to form the final product, γ-PGA. In the production of γ-PGA by *B. subtilis*, both the D- and L- forms of glutamic acid are present in the polymer, with a ratio of around 50-70% D-Glutamic acid to 50-30% L-Glutamic acid. This specific ratio is due to the activity of the Glutamate Racemase enzyme in the B. subtilis (natto) strains. Additionally, it has been noted that the Mn2+ ion plays a crucial role in regulating the (D:L) ratio during γ-PGA biosynthesis (**Bajaj *et al.*, 2011; Ashiuchi and Misono, 2002**). The biosynthesis of PGA occurs in several distinct stages, including racemization, polymerization, regulation, and degradation:

Racemisation

A problem that emerges during the manufacture and utilization of γ-PGA involves racemization. This term pertains to the conversion of one chiral isomer, or enantiomer, of a molecule into its mirror image. γ-PGA undergoes racemization when its L-glutamic acid units are transformed into their D-form, leading to the formation of both L- and D-enantiomers. γ-PGA can undergo racemization via different pathways, such as thermal processing, enzymatic action, and chemical modification. One possible cause of racemization is exposure to elevated temperatures during manufacturing or storage, which can affect the L-glutamic acid units (**Wang *et al.*, 2022; Wu *et al.*, 2006; Ashiuchi *et al.*, 2004**). Additionally, enzymatic racemization can happen when specific enzymes called racemases are present, and they have the ability to convert L- and D-enantiomers of amino acids (**Bajaj *et al.*, 2011**). The existence of D-enantiomers in γ-PGA can influence its characteristics and efficacy since the two enantiomers may display distinct biological activities and interactions. For instance, certain researches have indicated that D-γ-PGA demonstrates superior solubility and emulsifying capabilities when compared to L-γ-PGA. However, the presence of D-enantiomers may also cause decreased biocompatibility or heightened toxicity in some applications. To address the problem of γ-PGA racemization, different methods have been investigated, such as utilizing specific bacteria strains that exclusively produce L-γ-PGA, adjusting production conditions to decrease racemization, and employing purification techniques that can separate L- and D-enantiomers (**Candela and Fouet, 2006; Luo *et al.*, 2016**).

Polymerization

Polymerization of γ-PGA occurs through the action of γ-PGA synthase (pgs), which is a membrane-bound enzyme that catalyzes the polymerization of glutamic acid monomers into long chains of γ-PGA (**Ashiuchi *et al.*, 2001**). The process of polymerization relies on ATP, as demonstrated by research. ATP hydrolysis, which is dependent on the substrate, transfers the phosphoryl group of ATP to a terminal carboxyl group of elongated γ-PGA, according to (**Sung *et al.*, 2005; Ogunleye *et al.*, 2015**). The polymerization process is initiated by the activation of γ-PGA synthase, which requires the presence of Mg^{2+} ions and ATP. Once activated, the enzyme catalyzes the transfer of glutamic acid monomers from ATP to the growing chain of γ-PGA, forming a peptide bond between the carboxyl group of one glutamic acid and the amino group of another (**Najar and Das, 2015**). Polyglutamate synthase (pgs) is an enzyme that produces polyglutamate and is coded by four genes called pgsB, C, A, and E. In *Bacillus* species, ywsC, ywtAB, and capBCA are homologs of these genes (**Ashiuchi *et al.*, 2001, Sung *et al.*, 2005**). A recent study has identified pgsBCA as the only mechanism responsible for synthesizing γ-PGA in the active site of the synthase complex (PgsBCA), utilizing ATP as an energy source. The catalytic site mainly consists of PgsB and PgsC, while PgsA removes the elongated chain from the active site, which is necessary for adding the next monomer and transporting γ-PGA across the dense cell membrane (**Buescher and Margaritis, 2007**). According to a study, pgsE's involvement in γ-PGA production is not necessary, and high levels of pgsB, pgsC, and pgsA can produce γ-PGA even in the absence of pgsE. However, other research has shown that pgsE is crucial for the production of γ-PGA in the presence of Zn2+ in B. *subtilis*. This could be due to the instability and hydrophobic nature of the unique membrane-bound PgsBCA complex, which could make its isolation difficult (**Ogunleye *et al.*, 2015; Yamashiro *et al.*, 2011**).

Regulation

The regulation of γ-PGA production is controlled by multiple signal transduction systems, including the ComP-ComA regulator and the two-part DegS-DegU, DegQ, and SwrA system.The ComP-ComA regulator is a two-component signal transduction system that senses environmental signals such as quorum sensing molecules, and regulates gene expression in response to those signals (**Ohsawa et al., 2009**). The ComP-ComA system is involved in the regulation of γ-PGA production. When the ComP sensor detects high cell density, it activates the ComA transcription factor, which then activates the pgsB gene. The pgsB gene encodes for the γ-PGA synthase enzyme, which catalyzes the synthesis of γ-PGA.The two-part DegS-DegU, DegQ, and SwrA system is another signal transduction system that plays a crucial role in the regulation of γ-PGA production (**Calvio et al., 2008**). This system consists of a membrane-bound sensor kinase, DegS, and a cytoplasmic response regulator, DegU. In response to environmental signals, DegS phosphorylates DegU, which then activates the transcription of the pgsB gene. The DegQ protease and the SwrA protein also play important roles in this system. DegQ protease is responsible for cleaving and activating the DegU protein, while SwrA acts as a co-activator for DegU, enhancing its ability to activate gene expression (**Stanley and Lazazzera, 2005; Okada et al., 2005**).

Degradation

Researchers have been studying the enzymes that can degrade γ-PGA. Two such enzymes have been identified in *Bacilli*: endo-γ-glutamyl peptidase and exo-γ-glutamyl peptidase (**Obst and Steinbüchel, 2004**). Endo-γ-glutamyl peptidase is an enzyme that can break down the γ-PGA molecule by cleaving the peptide bonds between the glutamic acid residues. This enzyme is typically found intracellularly in Bacilli, meaning that it is produced inside the cell and works to break down γ-PGA molecules that are no longer needed. Exo-γ-glutamyl peptidase, on the other hand, is an extracellular enzyme that is secreted by Bacilli into the surrounding

environment (**King et al., 2000**). This enzyme can also break down γ-PGA by cleaving the peptide bonds between the glutamic acid residues, but it does so from the end of the molecule rather than in the middle. The degradation of γ-PGA by these two enzymes is important for several reasons (**Luo et al., 2016**). First, it allows *Bacilli* to recycle γ-PGA that is no longer needed, thereby reducing the accumulation of this polymer inside the cell. Second, it provides a potential avenue for researchers to modify the properties of γ-PGA by controlling the activity of these enzymes. By modulating the expression or activity of endo-γ-glutamyl peptidase or exo-γ-glutamyl peptidase, researchers may be able to reduce the viscosity of γ-PGA for improved processing or create new γ-PGA derivatives with unique properties. The discovery of endo-γ-glutamyl peptidase and exo-γ-glutamyl peptidase in Bacilli has shed light on the mechanisms by which these bacteria can degrade γ-PGA. Further research in this area could lead to new industrial applications for this biopolymer or provide insights into how to modify its properties for specific uses (**Kimura et al., 2004; Tran et al., 2000; Do et al., 2011**).

Microbial producers of γ-PGA

γ-PGA is a substance that is produced by a range of microorganisms, including bacteria, archaea, and eukaryotes. Some examples of these microorganisms include *B. anthracis, B. subtilis, B. licheniformis, B. megaterium, Bacillus pumilus, Planococcus halophilus, Sporosarcina halophile, Staphylococcus epidermidis, Natrialba aegyptiaca,* and *Hydra*. Scientists have noticed that γ-PGA production is limited to a particular group of bacteria, namely Gram-positive bacteria in the Order *Bacillales* and Class *Bacilli*. As a result, these bacteria are thought to be linked to each other from an evolutionary perspective (**Shih et al., 2001; Candela and Fouet, 2006; Oppermann-Sanio and Steinbüchel, 2002**) Table 1 presents a summary of the comparison of γ-PGA yield from different microbial producers.

Table 1 Comparison of γ-PGA yield from different microbial producers

Microbial Producer	Strain/bank number	Optimal Growth Conditions	Findings	References
B. subtilis ZC-5	CICC 20646	Relative humidity of more than 80% at 37 °C for 48h.	Yield of γ-PGA increased to 6.57 ± 0.13%	**Zhang et al., 2019**
B.subtilis Z15	CICC 20643	Agitation rate, temperature, and duration of growth, were established at 200 rpm, 37 C, and 48h, respectively.	Yield of γ-PGA was 42.92 ± 0.23 g/L	**Zhang et al., 2018**
B.*licheniformis* A14	MH763850	30 C for 24 h	Yield of γ-PGA was 37.8 g/L	**Mahaboob Ali et al., 2020**
B. licheniformis	NCIM 2324	200 rpm, 96 h, at 37±2°C	35.75g/L	**Bajaj and Singhal, 2009**
B.subtilis 242	CGMCC23791	at 37 °C for 12 h with agitation at 200 rpm.	32.14 g/L	**Li et al., 2022**
B. licheniformis P-104	HQ202723	Bioreactor, pH 7, 37 °C	Yield of γ-PGA was 41.6	**Zhao et al., 2013**
B. subtilis HB-1	CGMCC 1250	Bioreactor, pH 6.5, 37 °C	28.15	**Zho et al., 2014**
B. methyotrophicus	SK 19.001	pH 7.2, 37 °C	35.34	**Peng et al., 2015**
B. subtilis MJ80	CGMCC 3450	Fermenter for immobilized cell fermentation, pH 7, 37°C	68.7	**Ju et al., 2014**
		APFB (aerobic plant fibrous-bed bioreactor) immobilized cell fermentation, pH 7, 32°C	71.21	**Xu et al., 2014**
B. subtilis NX-2	CGMCC 0833	Bioreactor, pH 7, 32 °C	52.1	**Zhang et al., 2012**

γ-PGA can be produced through two different fermentation processes: solid-state fermentation (SSF) and submerged fermentation (SmF). Both methods have their advantages and disadvantages, and the choice of process depends on the desired yield, product quality, and cost-effectiveness. In Solid-State Fermentation (SSF), microorganisms are cultured on a solid substrate without any free-flowing liquid medium (**Sirisansaneeyakul et al., 2017**). The microorganisms obtain their nutrients from the solid matrix, and the moisture content of the substrate is maintained at a level that allows the growth of microorganisms (**Ashiuchi et al., 2001; Bajaj et al., 2011**). The process of PGA production through SSF involves using microorganisms, such as Bacillus subtilis, on a solid substrate, such as rice bran, wheat bran, or corn cob. The microorganisms grow on the substrate, and during their growth, they produce PGA. The PGA is then extracted from the solid substrate and purified. SSF has several advantages over SmF, including higher product yield, lower cost, and simpler downstream processing. However, the process has some disadvantages, including difficulty in controlling the fermentation conditions and maintaining consistent moisture levels throughout the substrate. In Submerged Fermentation (SmF), microorganisms are cultured in a liquid medium, and the fermentation process takes place in a fermenter. The

microorganisms obtain their nutrients from the liquid medium, and the fermentation conditions, such as temperature, pH, and aeration, are closely controlled. The process of PGA production through SmF involves using microorganisms, such as *Bacillus subtilis*, in a liquid medium containing a source of carbon, nitrogen, and other essential nutrients (**Bellon-Maurel et al., 2003**). The microorganisms grow in the liquid medium and produce PGA, which is then extracted from the medium and purified. SmF has several advantages over SSF, including better control over fermentation conditions, higher product purity, and the ability to scale up production. However, the process has some disadvantages, including higher cost and lower product yield. The production of PGA can be carried out through two different fermentation processes: solid-state fermentation (SSF) and submerged fermentation (SmF). Both methods have their advantages and disadvantages, and the choice of process depends on the desired yield, product quality, and cost-effectiveness. While SSF is more cost-effective and yields higher product quantities, SmF is more controlled and yields a purer product. Therefore, the selection of a process depends on the specific requirements of the application (**Bajaj et al., 2009**).

Characterization of γ-PGA

In order to fully understand the characteristics of γ-PGA, several analytical techniques are commonly used, such as Fourier-transform infrared (FTIR) (**Slavić et al., 2023**), high-performance liquid chromatography (HPLC) (**Guo et al., 2023**), nuclear magnetic resonance (NMR) spectroscopy (**Guan et al., 2023**), molecular mass determination (**Shi et al., 2023**), and amino acid analysis (**Pariyar et al., 2022**). FTIR spectroscopy is a powerful analytical technique that is used to identify functional groups in a sample (**Valand et al., 2020**). It is particularly useful in characterizing the chemical structure of biopolymers such as γ-PGA. The FTIR spectrum of γ-PGA shows characteristic peaks that correspond to various functional groups such as amide bonds, carboxyl groups, and C-H bonds. These peaks can be used to identify the presence and relative abundance of these functional groups in the γ-PGA molecule (**Xavier et al., 2020**). The functional groups of the produced γ-PGA from *Bacillus licheniformis* were investigated through FT-IR analysis by (**Xavier et al., 2020**). The resulting spectrum of *B. licheniformis* after 48 h fermentation displayed peaks at 3354 cm^{-1}, 1649 cm^{-1}, 1369 cm^{-1}, 1147 cm^{-1}, and 854 cm^{-1}, which confirmed the presence of OH bond, amide I band, C=O symmetric carbonyl stretch, C-N stretch, and C-H stretch in the synthesized polymer. The hydroxyl peak observed at 3354 cm^{-1} could be attributed to the bound water present in γ-PGA. Additionally, the presence of extra peaks at 1649 cm^{-1}, corresponding to amide I N-H bending, verified that the polymer synthesized was indeed γ-PGA (**Xavier et al., 2020**). HPLC is a chromatographic technique that is used to separate different compounds in a sample (**Hameedat et al., 2022**). In the case of γ-PGA, HPLC can be used to separate the individual polyglutamic acid chains based on their molecular weight. This information can be used to determine the average molecular weight of the γ-PGA sample, which is an important parameter that affects its physical and chemical properties. In their study, **Wang et al., (2019)** investigated the production of γ-PGA from *Bacillus siamensis* SB1001. They used HPLC to analyze the acid hydrolysate of the purified γ-PGA and observed two retention times of 43.47 min. (L-glutamic acid) and 46.34 min. (D-glutamic acid). The peak area ratio of L-glutamic acid was determined to be approximately 10.29%, indicating that the γ-PGA derivatives from *B. siamensis* SB1001 consisted of 10.29% L-isomer and 89.71% D-isomer glutamic acid (**Wang et al., 2019**).

NMR spectroscopy is a powerful analytical technique that is used to determine the three-dimensional structure of molecules. In the case of γ-PGA, NMR spectroscopy can be used to determine the precise arrangement of the glutamic acid residues in the polymer chain. This information is important for understanding the chemical and physical properties of γ-PGA, as well as for optimizing its production and processing. The focus of the study was on examining a bacterium, *Bacillus subtilis* C1, that has the ability to generate a bioconjugate of glycerol and g-PGA (**Shih et al., 2005**). The ^{1}H NMR spectrum was used to analyze the chemical shifts of both glycerol and g-PGA. The peaks observed in the range of 1.8-2.1, 2.3-2.4, 4.1-4.2, and 7.8 matched with those previously obtained from synthesized γ-PGA. Meanwhile, the peaks in the range of 3.50-3.54, 3.60-3.63, and 3.72-3.77 matched with the position of glycerol. The persistence of glycerol peaks even after dialysis through a membrane with 10 kDa cutoff for 3 days suggested that glycerol was covalently bound to γ-PGA. However, the glycerol peaks vanished after mild hydrolysis in 0.1% HCl solution at 150°C for 30 minutes, followed by dialysis and freeze-drying. Based on the 1H NMR spectrum, the ratio of γ-PGA to glycerol in the bioconjugate was roughly 10:1 (**Shih et al., 2005**).

The UV spectrum of γ-PGA was analyzed using a newly developed, uncomplicated quantitative method, allowing for more extensive research on the subject (**Zeng et al., 2012**). The technique relies on the UV absorption pattern of γ-PGA in water, which has a highest absorption wavelength of 216 nm. The findings from this method were similar to those from the high-performance liquid chromatography (HPLC) technique reported previously. This new method yields a linear calibration curve between 20-200 g/ml with a correlation coefficient of 0.9997. The precision (%R.S.D. < 1.50) and recovery (%R. > 99.29%) are satisfactory. The detection limit (LOD) and quantitation limit (LOQ) are 0.39 and 1.19 g/ml, respectively. These results were in agreement with the HPLC method (**Zeng et al., 2012**)

The scientists utilized Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS) analysis to ascertain the chemical makeup of the γ-PGA polymer that was generated by the NRC 20 bacterium (**Tork et al., 2015**). They discovered that the γ-PGA polymer consisted entirely of glutamic acid, and they detected peptide fragments of various lengths that were made up of glutamic acid residues. This provides strong support for the hypothesis that γ-PGA is derived from glutamate. Furthermore, the LC-ESI-MS analysis only identified polypeptides that contained glutamic acid in the reaction mixtures (**Tork et al., 2015**). Molecular mass determination is another important analytical technique for characterizing γ-PGA. There are several methods available for determining the molecular mass of γ-PGA, including size exclusion chromatography (SEC) (**Ikeda et al., 2018**), mass spectrometry (MS), and gel electrophoresis. These methods can be used to determine the average molecular weight, molecular weight distribution, and polydispersity of γ-PGA. γ-PGA from *Bacillus licheniformis* CGMCC 2876 was characterized by the high-resolution mass spectrometry (Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) (**Yan et al., 2015**).

Applications of γ-PGA

The unique characteristics of γ-PGA have made it a highly valuable substance with a wide range of practical applications. Its biodegradability, edibility, and non-toxicity to humans are essential qualities that make it suitable for use in various human-related purposes. Figure 3 displays the applications of γ-PGA.
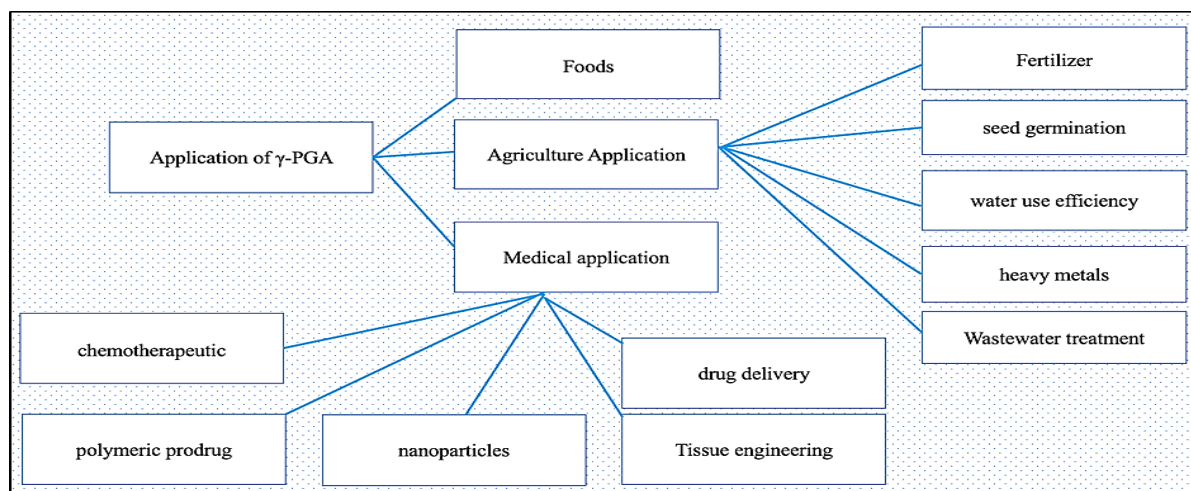

Figure 3 Application of γ-PGA

Food Applications

One of the main food applications of γ-PGA is as a thickener and stabilizer in various food products such as sauces, dressings, and beverages. It has been shown to improve the texture, mouthfeel, and overall quality of these products while also enhancing their shelf-life and stability (**Zagorska et al., 2022; Li et al., 2022**). The effects of γ-PGA at different levels (0.05, 0.1, 0.5 g/kg, w/w) on sponge cake properties were examined (**Shyu and Sung, 2010**). The addition of 0.5 g/kg PGA resulted in increased viscosity, emulsion stability, and foam stability. However, the enthalpy, onset, and peak temperatures of ice-melting transition of the sponge cake decreased significantly due to the addition of γ-PGA. The sponge cakes with 0.5 g/kg PGA were lighter in crumb color and white index compared to the control. Additionally, the internal structure of sponge cake with γ-PGA (0.1 and 0.5 g/kg) was finer and smoother than without. The incorporation of γ- γ-PGA also led to delayed staling of cake crumb and improved the texture of sponge cake (**Shyu and Sung, 2010**). Studies have shown that incorporating γ-PGA into milk-based drinks with polyphenols like cocoa, milk coffee, and milk tea can enhance their consistency and thickness. An experiment conducted by **Wang (2017)** investigated the effects of varying molecular weights of γ-PGA as a calcium-chelating agent in yogurt. The findings revealed that low-molecular-weight γ-PGA (300-400 kDa) could conceal the unpalatable flavor of metal ions in yogurt, while maintaining its acidity and pH. Moreover, it helped to delay the reduction of lactic acid bacteria in stored yogurt (**Wang et al., 2017**).

In addition to its use as a thickener and stabilizer, γ-PGA has also been investigated for its potential as a functional ingredient in various food products. For example, it has been shown to possess antioxidant, antimicrobial, and anti-inflammatory properties, making it a promising candidate for use in functional foods. In a laboratory setting (**Quach et al., 2022**), at a concentration of 4 mg/mL, pure γ-PGA demonstrated significant scavenging abilities against 1,1-diphenyl-2-picrylhydrazyl (with a reduction of $72.0 \pm 1.5\%$), hydroxyl (with a reduction of

81.0 ± 0.6%), and superoxide (with a reduction of 43.9 ± 0.8%) free radicals (**Quach *et al.*, 2022**). The γ-PGA polymer was examined for its ability to prevent the growth of several harmful microorganisms, including *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (**Ajayeoba *et al.*, 2019**). The researchers impregnated a paper disc with a concentration of 150 mg/ml of γ-PGA and tested it against these pathogenic bacteria, as well as *Salmonella enterica*, *Listeria monocytogenes*, and *Bacillus cereus*. The outcomes of the test that utilized paper discs revealed that the various pathogens exhibited varying degrees of sensitivity to the examined substances. In general, the γ-PGA polymer produced by the TA004 and TA006 isolates exhibited superior antimicrobial efficacy and resulted in zones of inhibition that were comparable to those achieved by standard antibiotics. More specifically, *S. aureus* and *L. monocytogenes* were more responsive to the compounds than *E. coli, Salmonella enterica*, and *P. aeruginosa*, whereas *Bacillus cereus* was fully unresponsive (**Ajayeoba *et al.*, 2019**). Table 2 presents a summary of the percentage of deactivation of various types of microorganisms. γ-PGA has the ability to conceal the unpleasant taste of potassium chloride, enabling the creation of a tasty, low-sodium salt that includes potassium chloride, as explained in reference (**Tanimoto *et al.*, 2010**). Additionally, **Wang *et al.*, (2017)** demonstrated that γ-PGA significantly reduces the bitter taste of various substances, including amino acids, peptides, minerals, vitamins, and caffeine. Moreover, γ-PGA has been found to enhance spiciness, prevent flavor deterioration, and maintain a harmonious flavor in food and drinks such as curry, potage soup, barbeque sauce, cola, and ginger ale. The presence of γ-PGA is believed to enhance the unsaturable transport in the lower intestine due to the resistance of its g-linkages to digestive enzymes.

Furthermore, when γ-PGA is added to foods high in minerals, it can speed up the absorption of minerals in the small intestine (**Tanimoto *et al.*, 2007**).

Furthermore, γ-PGA has been used as a carrier for the delivery of bioactive compounds such as vitamins and minerals. Its ability to form complexes with these compounds allows for their targeted delivery to specific parts of the body, enhancing their bioavailability and efficacy.

Table 2 Studies of γ-PGA effects on inactivation of microorganisms

Microorganism	Food Matrix/media	Findings	References
Escherichia coli *Bacillus subtilis* yeast	Cherries	The coating of γ-PGA was highly effective in slowing down the rate at which cherries spoil, delaying their ripening process, and extending their shelf life	**Yu *et al.*, 2023**
E. coli *B. subtilis*	aqueous solution	The newly produced Zn(γ-pga) compound exhibits strong antibacterial effects against *E. coli*, a gram-negative bacterium G$^-$, and *B. subtilis*, a gram-positive bacterium G$^+$.	**Akter *et al.*, 2021**
E. coli *S. aureus* *Salmonella enterica* *Pseudomonas aeruginosa* *Listeria monocytogenes* *B. cereus*	aqueous solution	The antimicrobial activity of γ-PGA polymer was found to be higher than that of conventional antibiotics. S. aureus and L. monocytogenes exhibited greater susceptibility than E. coli, Salmonella enterica, and P. aeruginosa. However, Bacillus cereus showed complete resistance to the polymer.	**Ajayeoba *et al.*, 2019**
S. enterotoxin A	pork meat	The use of γ-PGA/PLL nanoparticles loaded with nisin resulted in a notable reduction of Staphylococcal enterotoxin A	**Cui *et al.*, 2018**
Listeria monocytogenes *Salmonella typhimurium* *S.aureus* *Klebsiella pneumonia* *E. coli.* *Candida albicans*	aqueous solution	γ-PGA has the ability to prevent the growth of *L. monocytogenes, S. aureus, L. monocytogenes, S. typhimurium, K. pneumonia, E. coli*. The γ-PGA had a particularly strong inhibitory effect on S. aureus. However, there was no indication that the γ-PGA had any impact on the harmful yeast, C. albicans.	**Lee *et al.*, 2014**
E. coli *S. aureus*	aqueous solution	The γ-PGA showed its minimum inhibitory concentration (MIC) at 34 mg/ml for Staphylococcus aureus, but this value increased to 53 mg/ml for E. Coli. This suggests that a higher concentration of γ-PGA is required to inhibit G$^-$ bacteria compared to G$^+$ bacteria	**Bajestani *et al.*, 2018**
E. coli *S. aureus*	Nutrient Broth	chitosan and γ-PGA demonstrated bactericidal effects against both Escherichia coli and Staphylococcus aureus.	**Tsao *et al.*, 2010**
E. coli *S. aureus* *L. monocytogenes* *P. fluorescens* *P. putida*	fresh beef	The synergistic effects of γ-polyglutamic acid exhibited a stronger inhibitory effect on *E. coli, S. aureus, L. monocytogenes, P. fluorescens*, and *P. putida*	**Wang *et al.*, 2023**

Agriculture Application

Research has demonstrated that γ-PGA and other poly amino acids can enhance plant growth and nutrient uptake. γ-PGA can act as a substance that quickens fertilizer absorption and helps to retain fertility, while also improving the structure of soil and the capacity to hold water. Moreover, γ-PGA is a soil-friendly agent since it can be degraded by soil bacteria. Its usage can lead to a 10-30% increase in crop productivity while simultaneously reducing the amount of fertilizer needed by the same percentage. γ-PGA was effective in boosting tobacco yield and quality while decreasing the amount of fertilizer used for tobacco production (**Wang *et al.*, 2017**).

Chen's research indicated that g-PGA has the potential to boost the growth of pak choi by approximately 30% and improve the plant's nutrient uptake by roughly 25%. This effect may be attributed to g-PGA's ability to bind with cations and increase the active surface area of the plant's roots. **Wang *et al.*, 2007)** found that exposing tobacco seeds to various concentrations of g-PGA led to an increased germination rate, and the treated seeds showed higher levels of amylase and catalase activity compared to the control group. Furthermore, **Xu *et al.*, (2013)** mentioned that g-PGA could significantly enhance the growth of plants by promoting seed germination, increasing total biomass, and improving nitrogen uptake.

The use of γ-PGA resulted in a significant increase in the amount of water that the soil could hold when saturated, by 6.3-11.5% (**Shi *et al.*, 2023**). It also increased the field capacity by 8.4-15.3% and the amount of plant available water by 5.1-12.5% when compared to the control. Additionally, γ-PGA increased the amount of NO3⁻-N in the soil and the amount of residue, as well as improved the proportion of soil macro-aggregates when compared to the control. The application of γ-PGA also led to a substantial increase in winter wheat yield, ranging from 29.3-34.7%, and an increase in water use efficiency (WUE) of 21.2-33.3% compared to the control. Using γ-PGA at a concentration between 0.05-0.1% can be an effective method to enhance the physical and chemical properties of soil and increase the production of winter wheat in degraded soil (**Shi *et al.*, 2023**). Additionally, **Mohamed *et al.*, (2020)** confirmed the ability of γ-PGA to hold water in soil can be attributed to its high water retention capacity. It is capable of absorbing and retaining water up to 5000 times its weight, and the retention ability increases with the increase in its molecular weight (**Zhang *et al.*, 2004**).

Peng *et al.*, (2020) investigated the effectiveness of poly-γ-glutamic acid (γ-PGA) in removing heavy metals (Cu, Zn, Ni, and Cr) from contaminated farmland soil. The concentrations of heavy metals in the soil were Cu: 1180 mg/kg, Zn: 1450 mg/kg, Ni: 287 mg/kg, and Cr: 316 mg/kg. Batch experiments were conducted to determine the optimal washing conditions for maximum heavy metal removal. The γ-PGA concentration, washing time, liquid/soil ratio, and rotational speed were found to be the factors affecting the removal efficiency in order of importance. The optimal operating parameters were γ-PGA concentration of 3.5%, liquid/soil ratio of 15/1, washing time of 60 minutes, and rotational speed of 100 rpm, resulting in up to 50.7% removal of Cu. The soil contained heavy metals bound to Fe-Mn oxide and organic compounds. Treatment of the contaminated soil with the optimal washing conditions removed 54.3% of the Cu. The removal efficiency was further improved to 74.3% when the soil was washed three times with γ-PGA using the optimal parameters. The fact that the bacterial count in the soil was not significantly altered after using γ-PGA suggests that it is a safe and eco-friendly cleaning agent (**Peng *et al.*, 2020**).

Researchers examined the drought-resistant qualities of maize that had been treated with varying amounts of γ-PGA (0, 50, 70, and 100 mg/L) (**Ma et al., 2022**). The researchers monitored the plants' survival rates following a 7-day period of drought and discovered that the control plants experienced significant wilting and only a small portion (14.44%) were able to recover upon being watered again. Nevertheless, most of the maize plants that received γ-PGA treatment (ranging from 82.64% to 87.5%) were capable of speedy regeneration after being rehydrated, and there was no notable distinction in survival rates between the three γ-PGA concentration levels. These outcomes signify that even a lower dosage of γ-PGA, such as 50 mg/L, can have a considerable positive impact on the maize plants' drought tolerance (**Ma et al., 2022**).

Medical application

It is essential to optimize γ-PGA for drug delivery applications. The drug delivery properties of γ-PGA are dependent on its molecular mass, which has been identified as a critical factor. Controlling the release of drugs into tissues requires using polymers with different molecular masses. Nevertheless, studies have demonstrated that the molecular mass of γ-PGA is frequently higher than necessary for drug delivery applications (**Richard and Margaritis, 2006**). Chemotherapy's efficacy in treating cancer is often limited due to the harmful effects of drugs on healthy tissues. However, using polymer-drug conjugates can potentially address some of these issues. Many synthetic polymer-based anticancer drug conjugates are currently being tested in clinical studies. However, g-PGA stands out from the rest because it is made up of naturally occurring glutamic acid linked together through amide bonds, as opposed to a non-degradable C-C backbone like other polymers being tested. The repeating units of glutamic acid in g-PGA contain free carboxyl groups that enable drugs to attach to them. Due to these unique features, g-PGA has the potential to be an excellent carrier for polymer-drug conjugates that can selectively deliver chemotherapeutic agents (**Maeda, 2001; Li and Wallace, 2008**).

Phloridzin (PRZ) has the potential to be an effective antidiabetic drug because it can competitively inhibit glucose transport by binding to the Na^+/glucose cotransporter. However, it cannot be taken orally because the hydrolysis of a glucoside bond can release toxic phloretin. To solve this problem, a new conjugate called γ-PGA-PRZ was created. This conjugate was able to inhibit glucose transport in rats just as well as intact PRZ by preventing glucose transport from the mucosal to serosal sides of the everted small intestines. Therefore, γ-PGA can be a practical solution for creating a biodegradable polymeric prodrug that can be used as an oral drug delivery system by conjugating with a drug (**Xia et al., 2003; Ikumi et al., 2008; Wang et al., 2020**). Vaccine adjuvants play a crucial role in enhancing the immune response to a vaccine. A perfect vaccine adjuvant should be biologically harmless, have strong immunogenicity, and be easily obtainable. It should also efficiently transport antigens to antigen-presenting cells (APCs) and improve their ability to process and present antigens. This is necessary to stimulate the production of immunoregulatory cytokines. Although many vaccine adjuvants have been reported to trigger a robust antibody response, their ability to stimulate cellular immune responses is often limited. It is vital to develop an effective adjuvant that can induce both humoral and cellular immune responses that are specific to the vaccine antigens. In this regard, γ-PGA particles have been shown to be taken up by dendritic cells (DCs), which leads to the stimulation of cytokine production, upregulation of costimulatory molecules, and an enhancement of their ability to stimulate T-cells. Therefore, PGA is an attractive substance to consider for developing vaccine adjuvants. For instance, a study by **Uto et al., (2013)** explored the kinetics of antigen delivery using γ-PGA nanoparticles in mice after subcutaneous injection. They observed that DCs that capture γ-PGA nanoparticles migrate to the regional lymph nodes and effectively trigger strong cellular immune responses. (**Pathinayake et al., 2019; Uto et al., 2013; Yang et al., 2017; Okamoto et al., 2012**).

Tissue engineering involves creating biological alternatives to restore and maintain tissue function (**Jose Anju et al., 2018**). This is achieved by culturing cells on a scaffold to form tissue, which can be implanted in the patient's body. This approach overcomes immune response issues between donors and recipients. Polyglutamic acid is hydrophilic due to its anionic nature, making it unsuitable as a scaffold material. However, esterification of the -COO- groups to γ-PGA ethyl, γ-PGA -propyl, and γ-PGA -benzyl enhances its water resistance, making it a versatile material for tissue engineering. Among them, γ-PGA -Bn has better cell adhesion and viability than others. By attaching integrin-binding RGD peptide to unmodified -COO- groups, the scaffold can target integrin-binding mechanisms in cells. Electrospun γ-PGA -Bn scaffolds have thus been developed as a promising biomaterial for in situ human mesenchymal stem cell differentiation (**Jose Anju et al., 2018**).

Wastewater treatment of γ-PGA

γ-PGA presents a viable alternative for wastewater treatment that is eco-friendly due to its biodegradable and non-toxic nature. Compared to typical flocculants used in wastewater treatment plants following food processing fermentation processes, γ-PGA with a molecular weight of approximately 5.8-6.2×10^6 Da is thought to be superior (**Bajaj and Singhal, 2011**). Furthermore, it is worth mentioning that γ-PGA with a molecular weight of 9.9×10^5 Da was able to effectively remove 98% of basic dyes from an aqueous solution at a pH of 1 and can be reused, making it even more impressive (**Inbaraj et al., 2006; Luo et al., 2016**). Several researchers have examined the potential of γ-PGA for treating wastewater. Their studies have demonstrated that the addition of multivalent cations such as Ca^{2+}, Fe^{3+}, and Al^{3+} can enhance the flocculation ability of γ-PGA (**Shih and Van, 2001**). Moreover, γ-PGA derived from B. licheniformis was utilized to absorb Cu^{2+} ions, which served as a representative model for heavy metal ions found in industrial wastewater. Results indicate that γ-PGA has a strong affinity for Cu2+ ions and is capable of binding to them effectively (**Mark et al., 2006; Buescher and Margaritis 2007**).

Taniguchi et al., (2005) examined the ability of cross-linked poly-γ-glutamic acid (C-L γ-PGA) to flocculate bentonite, diatomaceous earth, *Escherichia coli*, *Mycrocystis aeruginosa*, crystal violet, and bisphenol A in polluted water samples from rivers and ponds. In some cases, pretreatment with polyaluminum chloride (PAC) was necessary to achieve the desired effect. Polluted water is typically composed of clay minerals, microorganisms, and chemical compounds. The mechanism of action of C-L γ-PGA is thought to involve electrostatic interaction between the flocculants (C-L γ-PGA and PAC) and the surfaces of the pollutants, resulting in the neutralization of their zeta-potential. The results of the study demonstrated that C-L γ-PGA was effective at purifying polluted water by flocculating and precipitating the contaminants (**Taniguchi et al., 2005**).

The impact of γ-PGA on the survival of probiotic microorganisms

The weight of PGA fractions is the primary factor in determining their uses. These uses include effective bioflocculant properties for fractions weighing over 2000 kDa, removal of metals and dyes for fractions between 2500-100 kDa, probiotic protectants for fractions weighing 300 kDa, and drug delivery tools for fractions weighing 50 kDa (**Nair et al., 2021; Bajaj et al., 2011; Mark et al., 2006; Ye et al., 2006**).**Bhat et al., (2015)** investigated the potential of bacterial poly-γ-glutamic acid to enhance the viability of probiotic bacteria and to examine how immobilizing γ-PGA would impact the survival of probiotic bacteria when they are stored in acidic fruit juice. *Bifidobacteria longum* and *Bifidobacteria* breve strains were placed on 2.5% γ-PGA and found to have a significantly better chance of survival (p< 0.05) in orange and pomegranate juice for 39 and 11 days, respectively, compared to when they were not immobilized. The survival rate of cells was significantly higher (p< 0.05) in orange juice than in pomegranate juice. Additionally, both *Bifidobacteria longum* and *Bifidobacteria breve* strains were able to survive in simulated gastric juice (with a pH of 2.0) when protected with 2.5% γ-PGA, showing a slight decrease (< 0.47 log CFU/ml) or no significant decrease in viable cells after 4 hours. On the other hand, free cells died within 2 hours in the same conditions. Therefore, γ-PGA had the potential to shield *Bifidobacteria* cells in fruit juice and may also facilitate their survival during the challenging conditions of the gastrointestinal tract (GIT) (**Bhat et al., 2015**).

Currently, researchers from around the world are investigating how γ-PGA can act as a cryoprotectant during the freeze-drying of probiotics (**Xavier et al., 2020**). These studies were aimed to create dry powder forms of probiotics that can be stored for longer periods and transported more easily. Specifically, these researchers are using γ-PGA that comes from isolated *Bacillus licheniformis* to protect lactic acid-producing bacteria like *Bifidobacterium bifidum* NCDC 235, *B. adolescentis* NCDC 236, and a strain taken from commercially available yogurt. Through these experiments, the researchers found that combining γ-PGA with sodium alginate at 10% (w/v) (27 log CFU/ml) and sucrose alone (25 log CFU /ml) was more effective in protecting and preserving the cell viability of *Bifidobacterium bifidum* NCDC 235. Neat γ-PGA (16 log CFU /ml) and mannitol (21 log CFU/ml) also showed similar results during the drying process at a temperature of -80°C for 48 hours (**Xavier et al., 2020**).

The researchers used a type of bacteria called *Lactobacillus plantarum*, which is a probiotic. They covered the bacteria with a substance called poly-γ-glutamic acid (γ-PGA400) in concentrations of 0.1%, 0.25%, or 0.5% that is made by another type of bacteria called *Bacillus* sp (**Jang et al., 2019**). The goal was to test how well the encapsulated bacteria survived under different conditions that can occur during probiotic production and ingestion. They found that without encapsulation, the levels of L. plantarum decreased by 1.50 log colony forming units (CFU)/ml during freeze-drying. However, when encapsulated with 0.5% γ-PGA400, the decrease was only 0.19 log CFU/ml. In the absence of encapsulation, all *L. plantarum* bacteria died within an hour when exposed to SGJ with a pH of 2. However, when they were covered with 0.5% γ-PGA400, they demonstrated the highest level of survival, with a reduction of only 0.30 log CFU/ml (**Jang et al., 2019**).

Conclusion and Future perspectives of γ-PGA

This review highlights the importance and potential of poly-gamma-glutamic acid (γ-PGA) in various fields, including food, medicine, and agriculture. γ-PGA is a natural polymer that is produced by several microorganisms, and its properties depend on the production conditions. γ-PGA has several properties that make it useful for various applications, such as water retention, biofilm formation, and metal ion chelation. Its potential applications include food additives, wound

healing agents, drug delivery systems, and soil conditioners. Additionally, this review emphasizes the prospects for future research on γ-PGA, including the development of new γ-PGA -based products and the exploration of its potential in novel applications. In the future, we can expect to see an increased use of γ-PGA in various fields, especially in the food and medical industries. Additionally, advances in biotechnology and bioprocessing technologies may lead to the development of new methods for the production of γ-PGA, which will further enhance its commercial value. Overall, γ-PGA represents an exciting area of research with significant potential for various applications, and further exploration is needed to fully unlock its potential.

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Informed consent All authors take responsibility for the integrity of the work as a whole, from inception to finish this review.

Research involves human or animals participation This research work did not involve human participation and/or animals.

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