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The Efficacy of Stabilizers on Lactoperoxidase System's Antibacterial Activity and Stability: A Review

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Abstract

The lactoperoxidase System holds significant prominence as a pivotal protein within milk, assuming a defensive role in combating diverse strains of microorganisms, particularly those with deleterious implications for milk quality. The stabilization process of the lactoperoxidase enzyme offers numerous advantages and benefits within an academic context and allows for controlled modulation of the enzyme's effects. Stabilized enzymes have attracted considerable research attention due to their favorable attributes, including easy separation from reaction mixtures, and the potential for reusing enzymes from multiple sources, to find and improve valuable methodologies. This review summarized the role of stabilizers in enhancing the efficacy and stability of the lactoperoxidase system and antibacterial applications in cosmetics, pharmaceutical, food, and health industries. This enzyme possesses characteristics that make it an effective natural antibacterial agent, leading to the extension of the lifespan and improvement of the stability of diverse types of dairy products and milk. It is used especially in remote areas where farmers are not close to the market. The common findings confirmed the ability of stabilizers to enhance enzyme stability in unfavorable environmental conditions that result in the extension of the activity period of the enzyme. Therefore, this characteristic indirectly enhances the enzyme's remarkable effectiveness against bacteria. Finally, according to various studies, the main function of stabilizing agents is the enhancement of enzyme stability as well as the longevity of the enzyme. Overall, it seems the stabilizers have a supplementary function in improving the enzyme's ability to kill bacteria through increased enzymatic activity.

1. Introduction

The dairy industry holds significant prominence in numerous countries and assumes a crucial role in both human nutrition and livestock production within a nation (Amiri Falahiani et al., 2020; Beheshti-Maal et al., 2011). The lactoferrin, lactoperoxidase ((EC 1.11.1.7)), Lysozyme (EC 3.2.1.17) and (Late Blowing Defect) glucose amidase (EC 3.5.1.4) are the main component of

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milk's natural antibacterial compounds. In 1939, the compound mentioned above was first found in cow's milk and later separated from both cow's and human milk. (Król et al., 2010). Various factors, such as age, lactation stage, and feeding practices, have an impact on the concentration of lactoperoxidase enzyme in dairy and milk (Althaus et al., 2001) .The main factor in milk that contributes to its intrinsic antibacterial characteristics is LBD glucose amidase. The above-mentioned compounds are widely employed in various industries as well as in the field of medicine (Hancock et al., 2002). Lactoferrin, a member of the transferrin family, is an iron-binding glycoprotein commonly referred to as lactoferrin. It possesses a molecular weight of approximately 80 kilodaltons, and, its efficacy as an antimicrobial, antiparasitic, antiviral, anti-inflammatory, and anticancer agent has been substantiated (Giansanti et al., 2016). The LBD-glucose amidase, an enzyme that breaks down l-Lysine ε-aminocaproyl-d-glucose, and lysozyme have gained considerable interest and find wide application in various industrial and medical settings. The LBD (Late Blowing Defect) glucose amidase, an enzyme that breaks down l-Lysine ε-aminocaproyl-d-glucose, and lysozyme have gained considerable interest and find wide application in various industrial and medical settings. The usage of LBD glucose amidase is also employed as an indicator in the diagnostic assessment of mastitis. These substances have many beneficial properties such as reducing inflammation, fighting cancer, combating microbial infections, regulating immune system function, improving intestinal bacteria, increasing hematocrit levels, and alleviating respiratory conditions. In addition to their mentioned therapeutic benefits, these components are also useful within the food industry for food preservation and packaging, and in the art of cheese making (Ebrahimi et al., 2021; Tiwari et al., 2009; Welk et al, 2021; Wolfson and Sumner, 1993). The present study focused on investigating the potential of the milk lactoperoxidase enzyme and its antimicrobial activities and how stabilizers can be utilized to improve the effectiveness and durability of the lactoperoxidase system.

2. Lactoperoxidase system (LPOS)

Lactoperoxidase system (LPOS) as one of the most effective antimicrobial systems in dairy products has a very strong inhibitory effects on Gram positive pathogenic bacteria such as Streptococci, staphylococci and *Bacillus* spp. as well as Gram negative bacteria e.g. *Salmonella, Shigella, Escherichia* and other coliform bacteria (Wolfson and Sumner, 1993). To perform its intended purpose, this particular enzyme necessitates the concurrent existence of two additional components, specifically hydrogen peroxide H_2O_2 and thiocyanate ion (SCN). The combination of these three components makes up the lactoperoxidase system found in milk. The lactoperoxidase system present in milk plays a role in enhancing its stability and preventing spoilage (Björck, 1978). The lactoperoxidase enzyme effectively neutralizes a diverse array of microorganisms through the generation of antibacterial compounds and displaying its wide-ranging antimicrobial activity, while the lysozyme possesses its inherent antibacterial properties and finds applications in multiple sectors such as cheese production, fish meat preservation, and food packaging (Koksal et al., 2020). Moreover, the concentration of enzymes in milk differs significantly among different animal species (Ebrahimi et al., 2021).

2. 1. Lactoperoxidase enzyme structure and mechanism of action

The lactoperoxidase, a metalloenzyme, is made up of an iron element and consists of a chain of 612 amino acids, with an approximate weight of 78 kilodaltons (León-López et al., 2022) and has an isoelectric point of 9.6. It is defined by the existence of a heme (iron) component and consists of roughly 10% carbohydrates. The enzyme shows an identical structural composition and has 4-5 carbohydrate chains.

2.1.1. Investigating the active site of lactoperoxidase enzyme

Thiocyanate is a crucial substrate of the LPO enzyme, exhibiting a greater affinity for this enzyme in physiological conditions compared to anions such as iodine, bromine, and chlorine.

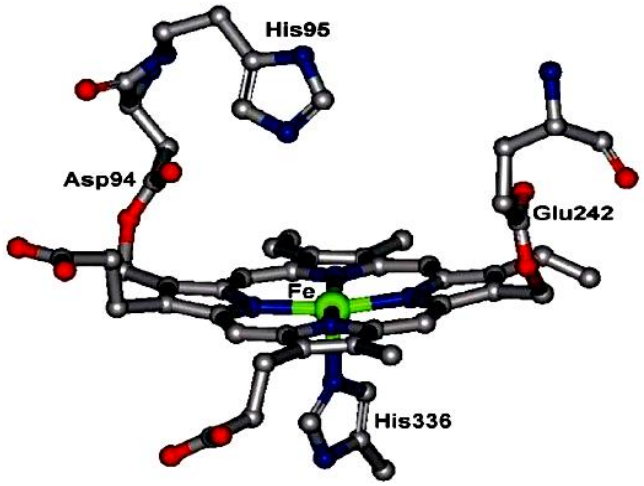
There are two hypotheses concerning the precise binding site of thiocyanate on the enzyme. One possible scenario entails the nitrogen portion of the thiocyanate ion binding to the iron component, while its sulfur portion forms a bond with the remote imidazole histidine ring (His 226). Furthermore, the participation of Arg amino acid 372 serves as a vital factor in enabling this binding process. Moreover, the engagement of Arg amino acid 372 is crucial in aiding this binding mechanism. Additionally, the presence of Arg amino acid 372 significantly contributes to the facilitation of this binding mechanism. Another hypothesis suggests that the maximum activity of the LPO enzyme may be influenced by the presence of the thiocyanate ion at a pH of 5.5. Additionally, at this pH range, the imidazole ring is expected to have a positive charge, enabling it to readily interact with the thiocyanate ion. The 372nd amino acid, arginine (Arg), is also believed to play a role in this activity by facilitating the connection between the SCN^- ion and the imidazole ring, i. e, the active site of the enzymeThe presence of each element in the LPO system has a particular role, resulting in the production of end products that possess antibacterial properties The LPO catalyzes reactions by utilizing hydrogen peroxide and halide ions, typically thiocyanate or iodide, as substrates. The reaction pathway involves several steps. Lactoperoxidase is activated by the addition of hydrogen peroxide (H2O2) and a halide ion (e.g., thiocyanate or iodide). This activation is crucial for LPO to exert its antimicrobial properties. LPO catalyzes the oxidation of the halide ions by hydrogen peroxide, resulting in the formation of hypohalous acids. For instance, with thiocyanate, hypothiocyanite (OSCN-) is formed, or with iodide, hypoiodite (OI-) is formed. These hypohalous acids, particularly hypothiocyanite or hypoiodite, act as powerful oxidants, disrupting microbial cell membranes, proteins, and nucleic acids. This oxidative stress leads to the inhibition of microbial growth, thereby preserving the integrity of the substrate (e.g., milk) or the environment in which the enzyme is acting. Then LPO itself undergoes a catalytic cycle where it is regenerated after catalyzing the reaction between hydrogen peroxide and the halide ions. It can repeatedly catalyze the conversion of these substrates, allowing for continuous antimicrobial activity. The precise details of the reaction pathway might vary slightly depending on the specific substrate and conditions, but the overall mechanism involves the activation of LPO, the generation of hypohalous acids, and the subsequent antimicrobial action against microorganisms through oxidative damage (Borjian-Boroujeni et al. 2021).

2. 1.2. Lactoperoxidase and mechanism of action

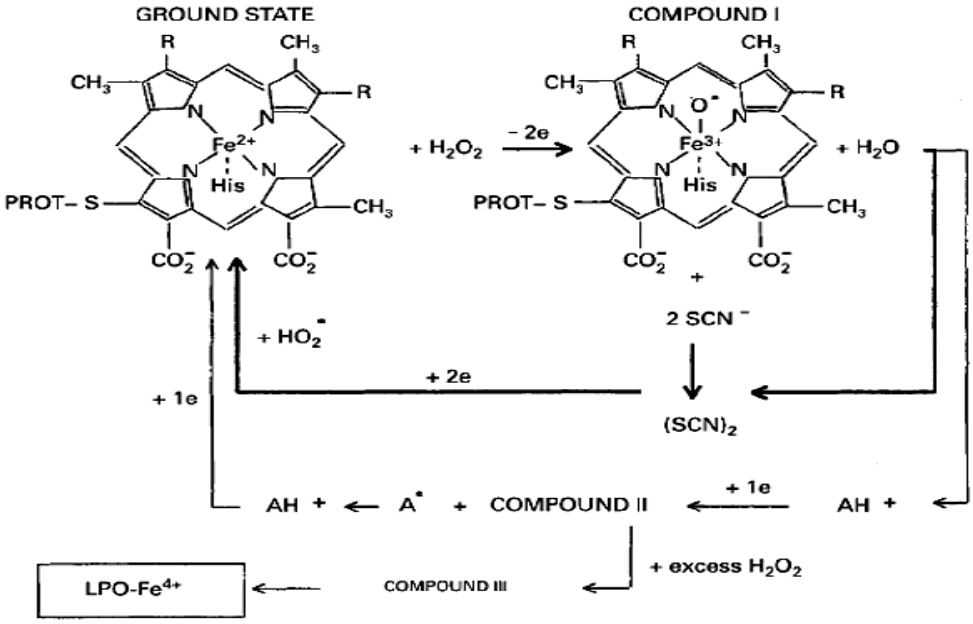
The lactoperoxidase enzyme exhibits bioactivity and possesses significant antimicrobial efficacy, rendering it a commonly utilized natural preservative in various domains. The LPO enzyme, known for its thermal stability, serves as a reliable marker for pasteurization. In milk, the LPO enzyme plays a significant role in combating detrimental bacteria, acting as a defensive protein. To perform its intended purpose, this particular enzyme necessitates the concurrent existence of two additional components, specifically hydrogen peroxide H_2O_2 and thiocyanate ion (SCN). The combination of these three components makes up the lactoperoxidase system found in milk. The lactoperoxidase system present in milk plays a role in enhancing its stability and preventing spoilage (Björck, 1978). The activity of the LPO enzyme remains preserved when subjected to conventional pasteurization procedures, specifically at temperatures of 63 °C for a duration of 30 min or 72 °C for a duration of 15 min. Figure 1 indiates the active site of LPO system(A) and its mechanism of action(B) (Al-Barrari et al., 2019; Gruden et al., 2023; Magacz et al., 2023)

Figure 1: The structure and the active site of LPO enzyme (A), and the reaction pathway of lactoperoxidase enzyme (B) Overall mechanism involves the activation of LPO, the generation of hypohalous acids, and the subsequent antimicrobial action against microorganisms through oxidative damage (Borjian-Boroujeni et al. 2021; Seifu et al, 2005; de Wit and van Hooydonk, 1996)

(A)

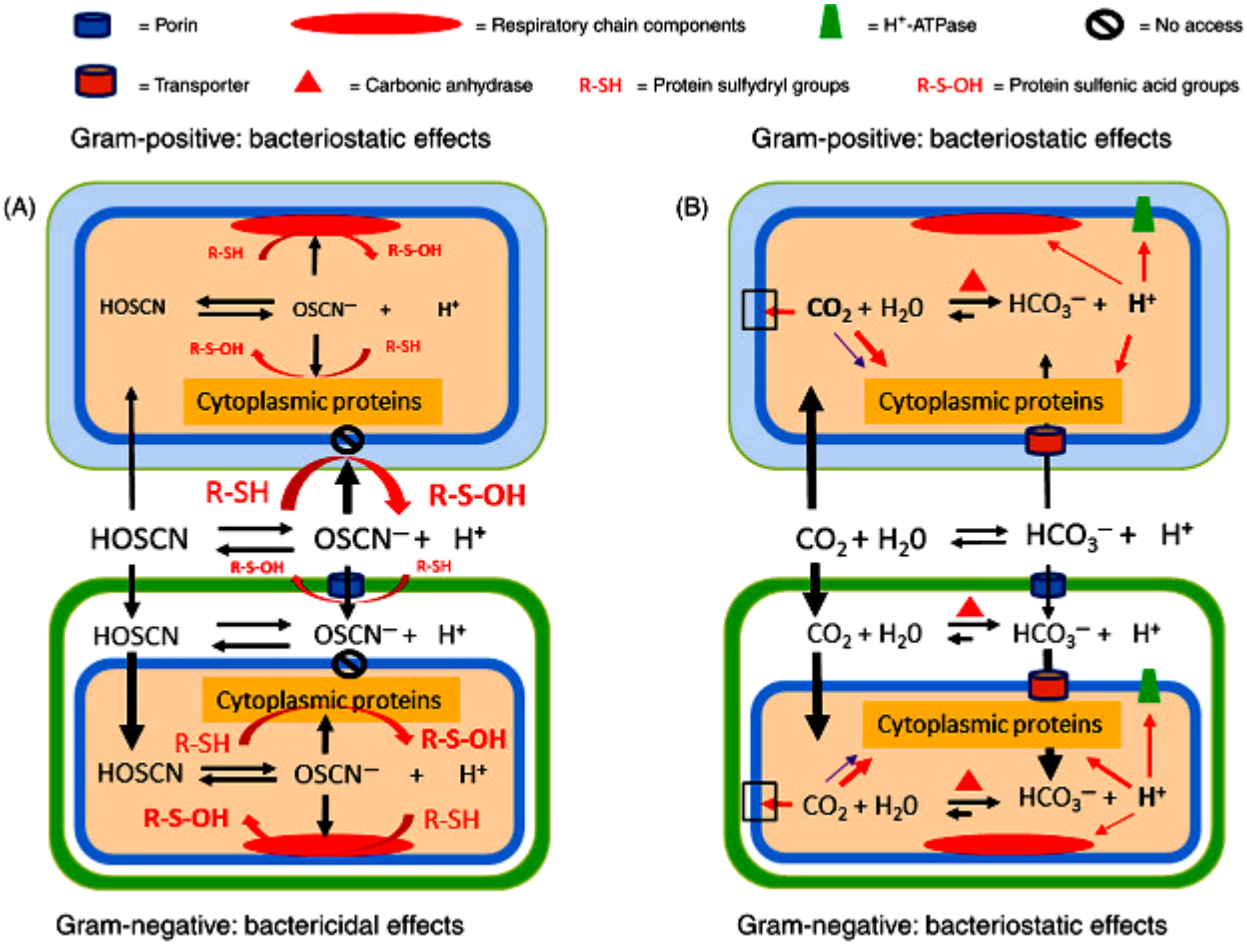


(B)



LPO has a glycoprotein composition and displays oxidoreductase capability. The LPO system consists of the lactoperoxidase enzyme, hydrogen peroxide, and thiocyanate, functioning as an innate antimicrobial mechanism. The system acts as a catalyst in the conversion process of thiocyanate to hypothiocyanate, which serves as an antibacterial agent. According to a study conducted in 2010, the system mentioned above demonstrated a 70% effectiveness in combating the *Staphylococcus aureus* bacterial strain. Furthermore, a series of investigations conducted in 2001 revealed significant observations and information concerning the capacity of this system to effectively minimize aflatoxin M1 levels. The system's ability to reduce toxins significantly improved by utilizing riboflavin. Furthermore, this specific system is utilized to maintain the quality of raw milk during the preservation process (Magacz et al. 2023).

Figure 2: Antibacterial Effects of Lactoperoxidase System (LPOS) and Contro the Gram negative and Gram-positive bacteria in bovine Raw milk. Lactoperoxidase is activated by the addition of hydrogen peroxide (H2O2) and a halide ion. **Formation of Hypothiocyanite or Hypoiodite:** LPO catalyzes the oxidation of the halide ions by hydrogen peroxide, resulting in the formation of hypohalous acids. **Microbial Inhibition:** The hypohalous acids, act as powerful oxidants, disrupting microbial cell membranes, proteins, and nucleic acids. This oxidative stress leads to the inhibition of microbial growth, thereby preserving the integrity of the substrate. **Catalytic Cycle:** During this process, LPO itself undergoes a catalytic cycle where it is regenerated after catalyzing the reaction between hydrogen peroxide and the halide ions. It can repeatedly catalyze the conversion of these substrates, allowing for continuous antimicrobial activity (Munsch-Alatossava et al., 2018).



2.2. The Mechanism of lactoperoxidase enzyme

The first step of all reactions catalyzed by heme peroxidases involves the conversion of H_2O_2, leading to the formation of compound I and altering the enzyme. Following this, the reaction pathway progresses with the involvement of appropriate substrates known as two-electron donors. In the event of modifications, the compound number I will revert to its initial enzymatic conformation. The progression of this reaction can be observed in (Fig 2). There are two distinct methods employed in the production of compound I. In the initial scenario, as commonly observed in the horseradish peroxidase plant enzyme, the oxidation of H_2O_2 involves the initial abstraction of an electron from the iron component of the heme group, followed by the subsequent abstraction of a second electron from the porphyrin ring. The first variant of compound number I is denoted as 1-cation. However, in the

instance of the cytochrome c peroxidase enzyme, a different version of compound number I arises, where the second electron is sourced from an amino acid neighboring the heme ring within the apoprotein. This specific form is referred to as the protein radical. In the presence of hydrogen peroxide, the lactoperoxidase enzyme transforms and produces 2 cations. The enzyme's iron component gives the first electron, while the porphyrin ring supplies the second electron, signifying the change. However, in instances where appropriate substrates are not present, this particular variant of the enzyme rapidly transmutes into a protein radical during the processes of isomerization and electron transfer from the amino acids of the apoprotein to the porphyrin ring. The involvement of tryptophan or tyrosine amino acids in this process holds considerable potential. The natural form of enzyme can be attained by compound number I through its oxidation process, which involves accepting electrons from compounds like SCN^- or halide compounds including I- and Br^-, as discussed in the preceding segment. It is imperative to acknowledge that the functionality of the electron transfer system hinges on the revitalization potential exhibited by these compounds. The composition of the second substrate plays a crucial role in determining the effectiveness of the H_2O_2 oxidation cycle facilitated by LPO. Specifically, the second substrate serves as an electron donor in this process. Due to this rationale and consequent outcomes, the approach is implemented in three distinct manners based on the inherent characteristics of the subsequent reactant involved in this process. The optimal activity of LPO as a two-electron donor can be observed when halides and thiocyanate are present at appropriate concentrations (Magacz et al., 2019; Hu et al., 1993; Chang et al., 1993). In the presence of non-optimal halide concentrations or single electron donors, the reaction pathway related to enzyme catalysis can be explained in the following way. Compound II is formed as a transitional compound when a single electron is transferred to compound I in the mentioned trajectory. The role of an inactive yet reversible form of the enzyme is taken on by this species. It is crucial to note that the conversion rate from compound number II to LPO is extremely low and practically insignificant (Kussendrager et al, 2000). Notably, the reaction between H_2O_2 and compound number II leads to the conversion of the compound into compound number III. Ultimately, this series of reactions culminate in the formation of the compound feryl lactoperoxidase (LPO [Fe IV]). The enzyme can be permanently deactivated, with its activity being reduced, and the length of time it remains inactive depends on the amount of H_2O_2, varying from minutes to hours. The LPO enzyme possesses remarkable significance in the composition of cow's milk. The preservation of raw milk is greatly aided by its profound antimicrobial properties, making it highly appealing. Raw milk has garnered considerable attention, with its observed lethal and restraining impacts on numerous bacterial strains in the milk being substantiated. The lactoperoxidase enzyme in bovine milk is known for its exceptional resistance to heat, making it one of the most thermos stable enzymes. The thermal pasteurization process, conducted at temperatures of 63 °C for a duration of 30 min or at 72 °C for a period of 15 seconds, only induces a minor level of deactivation in the microorganism under consideration. According to several investigations, it is suggested that to deactivate this enzyme in cow's milk, it is necessary to apply either a thermal process of 78 °C for 15 seconds or a thermal process of 80 °C for 2.5 second. However, the activity of the substance diminishes when it reaches a temperature of 80 °C for a duration of 2.5 seconds. The provided visual representation, denoted as (Fig 1) showcases the structure of LPO (Barrett et al., 1999).

3. Application of lactoperoxidase enzyme

The LPO enzyme is highly recognized and widely used in various industries due to its versatile functions and necessity as a crucial industrial compound in today's industrialized society. The lactoperoxidase enzyme is utilized in multiple industries, including the food industry, cosmetics industry, pharmaceutical industry, and laboratory kits (Jooyandeh et al., 2011).

3.1. Application of lactoperoxidase enzyme in food and dairy industries

Lactoperoxidase finds extensive use in the food industry for various applications. The primary use of LPO is predominantly seen in the food sector, especially when it comes to processing and storing dairy products (Jooyandeh et al., 2011). The LPO enzyme possesses characteristics that make it an effective natural antibacterial agent, leading to the extension of the lifespan and improvement of the stability of diverse types of food items, for instance, dairy products and milk. This effect is particularly beneficial in environments where the implementation of milk cooling mechanisms is not feasible. Furthermore, the utility of this enzyme can be extended to the manufacturing of a diverse range of canned goods. One additional function of lactoperoxidase is its ability to inhibit the formation of lactic acid in milk, consequently impeding milk sourness. Moreover, it also contributes to the reduction of yogurt acidity. Concentration of LPO at 5 ppm can notably diminish yogurt acidity. The LPO enzyme possesses the capacity to impede the proliferation of bacteria and effectively degrade them. The activity of the lactoperoxidase enzyme effectively inhibits the proliferation of gram-negative bacteria belonging to the catalase-positive group, such as *coliform, pseudomonas, shigella*, and *salmonella.* Additionally, under optimal environmental circumstances, this enzyme has the potential to induce their degradation. The deployment of lactoperoxidase has been seen to effectively hinder the multiplication of *E. coli* and *S. typhimurium* bacteria (Yousefi et al, 2022). Consequently, it has found application in formulations utilized in the manufacture of milk products intended for infants. Another prevalent utilization of LPO in the context of the food industry encompasses its incorporation in the production process of liquid extracts derived from carrots, tomatoes, chicken, and eggs. The aim was to tackle the presence of pathogens, namely salmonella enteritidis, in these specific food items. In addition, the evidence supporting the idea that LPO can hinder or neutralize this particular class of microorganisms has discussed (Jooyandeh et al., 2011; Sarikaya et al., 2015).

3.2. Application of lactoperoxidase system in cosmetics and health industries

The utilization of LPOs is progressively expanding and gaining momentum owing to its potent antimicrobial properties against bacteria, fungi, and yeasts. Within the cosmetics and healthcare sectors, this enzyme has found application in the form of emulsions or specialized creams. There has been significant modern interest due to its effectiveness and few negative effects, in comparison to other chemical compounds used for similar purposes. LPO has found numerous beneficial uses in the cosmetic-health field, including its significant contribution to the production and formulation of mouthwashes, enzyme toothpastes, and similar dental products. Another application worth mentioning is the incorporation of lactoperoxidase in the manufacturing and formulation processes of mouthwashes, enzyme toothpastes, and other dental materials within the cosmetic-health sector. One standout utilization of lactoperoxidase is its involvement in the manufacturing and formulation processes of mouthwashes, enzyme toothpastes, and other dental materials in the cosmetic-health industry. Bioten, a specific enzyme toothpaste, has been introduced to offer a concrete illustration (Magacz et al., 2019). The application of this specific toothpaste helps in averting the formation of dental plaques, as well as improving symptoms linked to common oral infections. The use of oral balance gel with LPO enzyme can effectively alleviate gum inflammation in people with salivary deficiency (Kussendrager et al., 2000) . The physiological role of human salivary lactoperoxidase (LPO) has explored, and a thorough analysis has conducted to compare the findings of clinical trials with those of in vitro investigations involving LPO alone and toothpaste enriched with bovine LPO. The functions of LPO reactivators and inhibitors and the possible use of nanoparticles to enhance the stabilization and efficiency of this enzyme have discussed (Magacz et al., 2019).

3.3. Application of lactoperoxidase enzyme in pharmaceutical industries

The LPO enzyme, combined with glucose oxidase and monoclonal antibody, offers a hopeful strategy for tumor control in the pharmaceutical field. The antiseptic properties of the Lpo enzyme prove its effectiveness and desirability for use in eye drop formulations for ocular applications. One notable application of the lactoperoxidase enzyme is its utilization in wound treatment, as well as antiviral activities. The products derived from the LPO system demonstrate the capacity to neutralize viruses, including the human immunodeficiency virus (HIV) associated with acquired immunodeficiency syndrome (AIDS), as well as viruses contributing to the development of cold sores (Sisecioglu, et al., 2010; Köksal and Alim, 2020)

3.4. Application of lactoperoxidase enzyme in laboratory kits

The LPO enzyme has potential applications in biosensors for quantifying iodine levels in blood and as a component in the enzyme-linked immunosorbent assay (ELISA) for antibody detection. An additional utilization of the LPO enzyme has observed in the production and assembly of laboratory kits, which serve the purpose of ascertaining the indomethacin half-life within the human body. The kits have accurately and efficiently measured the concentration of indomethacin in the bloodstream (Cooper, 2013, Urtasun et al., 2017).

4. Inhibition of bacterial growth by lactoperoxidase enzyme

Trammer and Wright had an important breakthrough in 1958 as they uncovered a relationship between the amount of lactoperoxidase (LPO) enzyme present in milk and its effectiveness against *streptococcus*. Additionally, subsequent research has revealed the significant roles hydrogen peroxide and thiocyanate play in the inhibition of bacterial growth through LPO activity. To effectively impede and halt the proliferation of bacteria, it is imperative to possess adequate measures that possess the capability to sufficiently inhibit their growth and development. As mention before, LPO does not possess germicidal activity, but in accombind with hydrogen peroxide and thiocyanate, its antibacterial effectiveness and functions as an antibacterial system work. This particular enzyme exhibits bactericidal properties towards Gram-negative bacteria, while demonstrating bacteriostatic effects towards Gram-positive bacteria (Bafort et al., 2014). The LPOS can effectively control the growth of a wide variety of susceptible microorganisms, comprising bacteria, fungi, and viruses, either by inhibiting their multiplication or by causing their death. The mechanisms of LPOS inhibition vary and are affected by various factors like test medium, electron donor type, temperature, pH, microorganism type, cell density, and incubation time (Bafort et al 2014; Silva et al, 2020; Yousefi et al, 2022).

5. The stability and activity of LPOS

Enzyme stabilization is instrumental in thwarting the contamination of protein/enzyme substrates and other compounds, thereby resulting in diminished expenses associated with purification procedures. The process of enzyme stabilization significantly enhances enzymatic stability and prolongs its half-life. This stabilization mechanism also facilitates the ability of enzymes to operate on a broader scale within larger ecological contexts, potentially enabling interactions with other enzymes. To enhance the application of enzymes in laboratory and industrial settings, as well as to facilitate their reusability in manufacturing, it is imperative to enhance enzyme stability. The process of stabilization has the potential to rectify the problem associated with enzyme instability. Immobilized enzymes offer notable advantages, including enhanced enzyme stability, the potential for enzyme reuse and reproducibility, and facile separation from the reaction mixture. These advantageous aspects have piqued the interest of researchers, consequently driving efforts towards expanding the methods of enzyme immobilization and broadening the scope of applications for immobilized enzymes. The predominant techniques for enzyme

immobilization encompass non-covalent adsorption, ionic interactions, covalent bonding, enzyme cross-linking, and entrapment within a polymer gel or capsule (Lotfi et al., 2014; Lotfi et al., 2015; Dawoodi et al., 2015; Nunes et al., 2010; Bostock et al., 2020; Guisan, 2006). The advantages of using enzyme immobilization are including stabilization of monomeric enzymes against decomposition, environmental changes, destructive bacterial and proteolytic agents, stabilization of enzymes in solutions especially organic solvents, against toxins, hydrogen peroxide and radical agents, the possibility of using enzymes in continuous systems, increasing the duration or lifespan of enzyme activity, the ability to use enzymes repeatedly and reduce the costs of using enzymes and finally ncreasing and expanding the activity of enzymes in some rare cases (Mohamad et al., 2015; Cass et al., 1998).

The stabilization of lactoperoxidase presents several noteworthy benefits within the field of enzymology, notably encompassing facile separation techniques, decreased production costs owing to the possibility of reuse, and the ability to regulate the enzyme's effect. Semsam Shariat and colleagues conducted a scholarly investigation with the aim of developing a simple and efficient method to improve the stability and activity of lactoperoxidase (LPO) by immobilizing it on graphene oxide nanosheets (GO-NS). In the current investigation, subsequent to the purification of lactoperoxidase (LPO) sourced from bovine whey, it was affixed onto functionalized graphene oxide nanosheets (GO-NS) by means of glutaraldehyde, acting as a binding agent. The examination extended to studying the kinetic characteristics and stability of both unbound and protected Lipid Peroxidation (LPO) compounds. The findings of the study indicated a considerable enhancement in the stability of stabilized LPO, as compared to its free form (Semsam Shariat et al., 2018).

Sheikh and his colleagues conducted a study to explore the potential of immobilizing Lipoxygenase (LPO) on silver nanoparticles (AgNPs) in order to improve the antimicrobial effectiveness of LPO. The formation of a conjugate called LPO/AgNP occurred when the enzymatic compound LPO was immobilized onto a substrate made of silver nanoparticles. In the ongoing investigation, scientists found that LPO was successfully immobilized onto silver nanoparticles to form the LPO/AgNP conjugate. The use of silver nanoparticles as a substrate led to the formation of the LPO/AgNP conjugate in the current study. The stabilized LPO/AgNP conjugate was subjected to rigorous analysis using a range of biophysical techniques to obtain its comprehensive characterization. The augmented antibacterial efficacy of the conjugate was evaluated by subjecting it to testing against *E. coli* in a cultured medium at intervals of two hours for a duration of ten hours. The experimental findings demonstrated the effective synthesis of spherically-shaped silver nanoparticles. The LPO enzyme was effectively immobilized onto silver nanoparticles (AgNPs) with an average agglomerate size of approximately 50 nm. The conjugate in its stabilized form demonstrated enhanced antibacterial efficacy against *E. coli*. The findings of this study reveal significant distinctions between coli in comparison to free LPO. By conducting this research, the effectiveness of the lactoperoxidase system could be improved, leading to the discovery of new methods to strengthen the stability and antimicrobial properties of the LPO system in the dairy industry and related field (Sheikh et al., 2018). A research was conducted to examine how the LPOS system and the hypothiocyanate ion affect the antibacterial properties of fresh milk when incubated at 30 °C for 24 hours. The results revealed that the introduction of this system to fresh milk resulted in a considerable reduction in bacterial proliferation, causing it to fall below the standard limit of 5.35×10^3 cfu/ml. Additionally, the pH of the fresh milk remained significantly stable throughout the incubation period, measuring at 6.475. This study involved the activation and development of the LPOS system in fresh milk. Following a 48-hour incubation period at 30 °C, the pH level remained stable at 6.8 and the proliferation of bacteria was effectively inhibited (Puspitarin et al., 2013). According to Villa et al's (2014) research, which examined the impact of the LPOS system on

bacterial growth in milk and its ability to enhance milk stability, it was determined that the addition of a hypothiocyanate-rich solution to milk did not cause any significant alterations in pH levels. Specifically, following a six-hour incubation period, no changes were observed, with the pH remaining constant at 6.66. Furthermore, after the designated period of incubation, the growth and advancement of all bacteria were also hindered, leading to a final count of 8±8.6 cfu/ml. Conversely, in milk devoid of a solution abundant in hypothiocyanate, after a duration of 6 hours in incubation, the milk's pH had declined to 5.5, suggesting an absence of milk stability. Additionally, the total bacterial count had increased (Villa et al., 2014). Furthermore, in the investigations conducted by Al-Baarri et al, findings in their study revealed that implementing a hypothiocyanate-enriched solution or activating the lactoperoxidase-thiocyanate-hydrogen peroxide system in milk effectively manages the proliferation of *Salmonella enteritidis* bacteria. This confirms the theory that the antibacterial effects in milk are a result of the properties of the hypothiocyanate ion. The experiment involved adding the aforementioned system to milk and creating a suspension that contains hypothiocyanate. Significant suppression of the growth of *Salmonella* and *Shigella* bacteria was observed as a consequence (Al-Baarri et al., 2010). In other research endeavor, the process of immobilizing lactoperoxidase onto silica-coated magnetite nanoparticles was undertaken with the aim of enhancing the enzyme's properties despite the presence of cadmium chloride, which acted as an inhibitor. The study revealed that the concentration of cadmium chloride exerts a direct influence on the activity of lipid peroxidation (LPO) and subsequently alters its kinetic parameters. Furthermore, the results showed that the immobilized version of LPO demonstrated greater durability compared to its unattached form. The results of this research suggested that when lactoperoxidase is immobilized on silica-coated magnetite nanoparticles, its stability increases in the presence of cadmium chloride as an inhibiting substance (Babadaie Samani et al., 2016). In other research, the lactoperoxidase system in milk was studied by using glucose oxidase immobilized in Electrospun Polylactide microfibers. The objective of their research involved the immobilization of glucose oxidase (GOX) within polylactide (PLA) fibers in order to facilitate the activation of the lactoperoxidase (LP) system in milk. Microfibers incorporating glucose oxidase (GOX) were produced by means of electrospinning, utilizing emulsions formed through dispersion of aqueous GOX in a solution of polylactic acid (PLA) dissolved in a mixture of chloroform and N, N-dimethylformamide. The enzymatic activity exhibited by GOX-in-PLA fibers with a diameter of 1100 ± 400 nm surpassed that of the GOX-in-PLA membrane prepared through direct casting, primarily because of the greater surface area offered by the electrospun fibers. The implementation of GOX-PLA fibers for the activation of LP in model solutions yields a more persistent production of antimicrobial $OSCN^-$ in comparison to direct activation utilizing H_2O_2. The assessment showed that electrospun GOX-in-PLA microfibers can stimulate the LP system. This suggests the potential of microfibers for food packaging to extend milk shelf life (Zhou and Lim, 2009). In 2010, Jafari and his collegues purified, stabilized, and examined bovine lactoperoxidase. In this study, the effectiveness of enzyme stabilization to improve enzyme properties was examined. In the study, lactoperoxidase purification was done using various chromatography techniques. The use of polyaniline polymer to immobilize lactoperoxidase is due to its unique properties. The initial step involved the activation of the polyaniline polymer through the use of glutaraldehyde, followed by the subsequent successful immobilization of lactoperoxidase onto the activated polymer. Next, the study delved into examining the impacts of enzyme concentration, duration, and pH levels on the efficiency of stabilization. The surface of both polyaniline and polyaniline-enzyme polymers were examined with an atomic force microscope. The binding efficiency was 91%, indicating that the immobilized lactoperoxidase retained a significant portion of its original activity. Additionally, research findings indicate a significant enhancement in the stability of immobilized enzymes as compared to enzymes

present in their free form. Furthermore, the immobilized enzyme exhibits the desirable characteristic of reusability, wherein it can be utilized multiple times without experiencing a decline in activity or a diminishment of its efficacy (Jafari et al., 2013). Welk et al focused on extensively examining the antibacterial and antiplaque efficacy demonstrated by a particular lozenge composition which includes lactoperoxidase, thiocyanate, and hydrogen peroxide. A lozenge is a small, often diamond-shaped or rounded, medicinal tablet or sweet that is typically flavored and dissolved slowly in the mouth. These are commonly used for soothing sore throats or as a form of candy or breath freshener. They come in various flavors like mint, fruit, or herbal extracts and often contain active ingredients like menthol, honey, or medicinal herbs. The lozenge is usually flattened and rounded at the edges, resembling a diamond. The findings of their study suggested that the incorporation of a comprehensive LPO-system within lozenges has the potential to effectively inhibit plaque regrowth and minimize the presence of cariogenic bacteria. Therefore, it is reasonable to include these lozenges as part of one's daily oral hygiene routine (Welk et al., 2021).

5.1. Optimizing Milk Preservation with Lactoperoxidase Systems

Researchers have examined the efficacy of lactoperoxidase systems (LPOs) as preservation agents and inhibitors of microbial growth at ambient storage temperatures. The findings demonstrated that the activation of LPs exhibited a statistically significant ($P<0.05$) effect in prolonging the shelf life by 8 hours. Activated milk exhibited titratable acidity (0.19) and a pH value (6.52). Conversely, control milk displayed a lower pH value (6. 24) and higher titratable acidity (0.21). The concentration of viable microorganisms, including bacteria, yeast, mold, coliform, and Staphylococcus, in activated milk exhibited a significant reduction ($p < 0.05$). After a duration of 6 hours, the control group exhibited a rise in the overall bacterial count to 7.36. Conversely, in the activated milk, the bacterial count reached 6.24 log cfu/ml, displaying a statistically significant reduction ($p < 0.05$) compared to the initial count. The application of LPs resulted in a reduction of the coliform count by 0.45 log log cfu/ml, whereas the control group exhibited an increase of 0.49 After 12 hours of storage, the count of Staphylococcus was reduced by 23.13% through the use of LPs. Linear programming models can be effectively employed to extend the shelf life and preserve the microbiological integrity of raw cow's milk in situations where there is a lack of cooling facilities (Awol et al., 2023).

5.2. Stabilization of LPO with osmolytes

The enzyme LPO stabilization by osmolytes such as ectoine, hydroxy ectoine and betaine have been investigated. The impact of ectoine, a compatible solute, has explored on the structural properties, thermal stability, thermodynamic parameters, enzymatic activity, and stability of LPO. The research results have showed that the presence of higher ectoine concentration led to an improvement in LPO's catalytic effectiveness. The phenomenon of ectoine's binding to the LPO was determined through research involving UV-visible absorption spectroscopy and FTIR spectra analysis. Furthermore, ectoine exhibited a significant augmentation in both the enzyme's transition temperature and Gibbs free energy. The fluorescence measurements demonstrated a significant attenuation in LPO fluorescence upon the addition of ectoine. The quenching mechanism likely involved static quenching through the formation of a ground state complex. The thermodynamic analysis revealed that hydrogen bonding and Vander Waals intermolecular forces were significant factors influencing the interaction process of LPO with ectoine. Ectoine's potential as a stabilizing agent for lactoperoxidase is highlighted by the results of this study, suggesting promising applications in both industrial and medical fields (Borjian-Boroujeni and Nayeri, 2018). In another study, the impacts of hydroxyectoine on LPO's stability and structural integrity were examined using additional investigations that incorporated methods like fluorescence spectroscopy, FTIR spectra, docking studies, and enzyme activity assay. The outcomes demonstrated that the

supplementation of hydroxyectoine resulted in a notable enhancement in the catalytic activity of LPO. The attachment of hydroxyectoine to the LPO was confirmed by utilizing FTIR and UV-visible spectroscopy methods. The utilization of fluorescence spectroscopy elucidated that hydroxyectoine exhibited quenching properties towards the fluorescence emitted by lipid peroxidation. The investigation of thermodynamic parameters and molecular docking analysis revealed that hydrogen bonding and van der Waals forces played prominent roles in addition to the elevation of the enzyme's melting temperature (Tm) (Borjian-Boroujeni and Nayeri, 2021). After adding the above-mentioned stabilizers to LPO, the properties of stabilized LPO as well as free enzyme under different environmental conditions were measured. The different ranges of pH, temperature and treatment times on enzyme activity and stability were evaluated. Two-way analysis of variance showed that both group and pH had a significant effect on enzyme activity ($P<0.001$). Among the groups, the highest level of activity was related to the ectoine group and the lowest level of activity was related to the control group. Regarding pH, the highest level of enzyme activity in the ectoine and hydroxyectoine groups were 6.4 and 4, respectively (Table 2) (Borjian-Boroujeni et al., 2018; Borjian-Boroujeni et al., 2021).

Table 2: The evaluation of LPO activity after stabilization with ectoine and hydroxyectoine in different pH

Group	pH	LPO Activity (IU/ml)	Standard deviation (IU/ml)
Control	4	2.87	0.46
	5.5	2.68	0.17
	6.4	2.92	0.24
	7.5	1.99	0.28
	8.5	0.84	0.17
	9.6	1.60	0.23
Ectoine	4	3.86	0.04
	5.5	2.86	0.28
	6.4	4.23	0.29
	7.5	2.39	0.01
	8.5	1.66	0.06
	9.6	3.91	0.26
Hydroxyectoine	4	4.46	0.51
	5.5	3.82	0.37
	6.4	2.27	0.11
	7.5	1.62	0.14
	8.5	1.56	0.09
	9.6	0.98	0.21

Three-way analysis of variance showed that group, time and concentration had a significant effect on enzyme activity ($P<0.001$). Regarding the group, the highest level of enzyme activity was in the ectoine group and the lowest level of activity was in the control group. Also, the level of enzyme activity in the hydroxyectoine group was significantly higher than the control group and lower than the ectoine group ($P<0.001$). Regarding time, the highest amount of enzyme activity in all three groups of ectoine, hydroxyectoine and control was at 48 hours (Table 3) (Borjian-Boroujeni et al., 2018, Borjian-Boroujeni et al., 2021).

Table 3: The evaluation of LPO activity after stabilization with ectoine and hydroxyectoine in different concentrations and incubation times

Group	Time	Concentration (molar)	Enzyme Activity (IU/ml)	Standard deviatian	Group	Time	Concentration (molar)	Enzyme Activity (IU/ml)	Standard deviatian
Ectoine	5h	0.05	2.34	0.13	Hydroxy Ectoine	5h	1	2.89	0.29
		0.3	3.23	0.21		24h	0.05	1.45	0.05
		0.8	3.31	0.68			0.3	2.92	0.26
		1	3.14	0.26			0.8	7.19	0.21
	24h	0.05	2.09	0.39			1	6.65	0.41
		0.3	6.77	0.37		48h	0.05	4.70	0.33
		0.8	7.75	0.23			0.3	5.66	0.34
		1	7.46	0.15			0.8	6.31	0.18
	48h	0.05	5.15	0.09			1	5.83	0.26
		0.3	5.60	0.62		72h	0.05	0.61	0.03
		0.8	7.28	0.19			0.3	0.49	0.03
		1	7.38	0.32			0.8	1.95	0.21
	72h	0.05	0.69	0.04			1	2.02	0.36
		0.3	1.82	0.23		1week	0.05	0.01	0.005
		0.8	2.43	0.22			0.3	0.007	0.002
		1	2.04	0.06			0.8	2.32	0.16
	1 week	0.05	0.25	0.01			1	3.47	0.05
		0.3	0.34	0.05					
		0.8	4.85	0.36	Ectoine (Control)	5h	-	2.04	0.1
		1	3.97	0.04		24h	-	4.54	0.37
Hydroxy Ectoine	5h	0.05	2.69	0.52		48h	-	4.96	0.35
		0.3	2.97	0.20		72h	-	1.7	0.03
		0.8	3.54	0.18		1week	-	0.04	0.04

5.3. LPOS-containing films and coatings

Lactoperoxidase-containing films and coatings are innovative materials designed with the integration of lactoperoxidase systems (LPOS) into their composition. Lactoperoxidase exhibits antimicrobial properties that can be harnessed for various practical applications, particularly in food preservation and packaging. These films and coatings are engineered to:

1. Preserve Freshness: LPOS-containing materials aim to extend the shelf life of perishable goods by inhibiting the growth of bacteria, fungi, and certain viruses that could otherwise cause spoilage or deterioration.

2. Antimicrobial Protection: The incorporation of lactoperoxidase, along with other components like hydrogen peroxide and thiocyanate or iodide ions, creates an antimicrobial environment that actively combats microbial growth, thereby enhancing the safety and quality of packaged products.

3. Enhance Shelf Stability: By actively suppressing the growth of microorganisms, these films and coatings help maintain the freshness, nutritional quality, and safety of products, especially those susceptible to rapid spoilage.

The development and utilization of LPOS-containing films and coatings involve various scientific and engineering methodologies. Researchers have focused on optimizing the concentration of lactoperoxidase, the proportions of additional components, and the compatibility of these materials with different types of packaging. They also have investigated the efficacy of these coatings under varying storage conditions to ensure their functionality and reliability. Studies and research in this area often

involved experimentation to evaluate the antimicrobial effects of LPOS-containing films and coatings. Researchers quantify microbial counts, monitor the shelf life extension of packaged goods, and assess the overall impact on product quality and safety. Furthermore, these materials are part of ongoing efforts to develop sustainable and eco-friendly packaging options. Incorporating lactoperoxidase into packaging materials aligns with the aim of reducing food waste by preserving perishable items for longer periods. Overall, LPOS-containing films and coatings represent an innovative approach in food packaging technology, leveraging the natural antimicrobial properties of lactoperoxidase to safeguard products and contribute to the development of more efficient and sustainable packaging solutions. Table 4 indicates the stability and antibacterial characteistics of LPOS before and after using various specific films and coatings (Gruden et al., 2023; Magacz et al., 2023).

Table 4: The stability and antibacterial properties of LPOS with/without different coatings and films

Pathogenes	**Impacts**	**Films and Coatings Containing Lactoperoxidase**	**References**
Escherichia coli	LPOS showed the greatest reduction of bacteria		Al-Baarri et al 2019
S. mutans *Lactobacilli*	The LPO-system-lozenge inhibiting plaque regrowth and reducing cariogenic bacteria		Welk et al 2021
Salmonella typhimurium DT 104	Inhibitory effect against bacteria	Soybean meal + LPOS	Lee and Min 2013
Salmonella enterica *E. coli* O157:H7	The films inhibited *S. enterica* and *E. coli* O157:H7	Whey protein films + LPOS	Min et al 2005a
L. monocytogenes	Whey protein coating containing LPOS inhibited *L. monocytogenes* in smoked salmon	LPOS+whey protein coating	Min et al 2005b
Pseudomonas spp. *Shewanella spp*.	Chitosan-LPOS films had the highest inhibitory activity against all bacterial growth	Chitosan-LPOS coating	Ehsani et al 2020
Gram-negative and Gram-positive bacteria	LPOS with essential oil to achieve an antibacterial activity for both Gram-negative and Gram-positive bacteria. The lowest count of *L. monocytogenes* was observed	Whey protein coating +LPOS (5% v/v) + Bunium persicum essential oil	Saravani et al. 2019
E. coli (NRRL B-3008), *Listeria innocua* (NRRL B-33314), *Pseudomonas fluorescens* (NRRLB-253)	The LPOS inhibited *L. Innocua* & *P. Fluorescens* for 24-h incubation. The most resistant bacteria to LPOS were *E. coli* and *P. fluorescens*.	Alginate-LPOS coating	Yener et al 2009
Enterobacteriaceae, *Pseudomonas aeruginosa*, aerobic mesophilic bacteria	The antimicrobial activity of coating containing LPOS was attributed to the interaction of $OSCN^-$ and HOSCN with SH groups of proteins in the cell membrane	Alginate-LPOS coating	Yousefi et al 2018
Aerobic mesophilic bacteria, *Enterobacteriaceae*, and *P. aeruginosa*	LPOS enhanced an antibacterial activity	Whey protein+alginate +lactoperoxidase	Molayi et al 2018
S. putrefaciens *P. fluorescens*	Whey protein coating + LPOS + MAP showed lowest bacterial count.	Whey protein coating + LPOS + MAP	Rostami et al 2017

Continue table 4

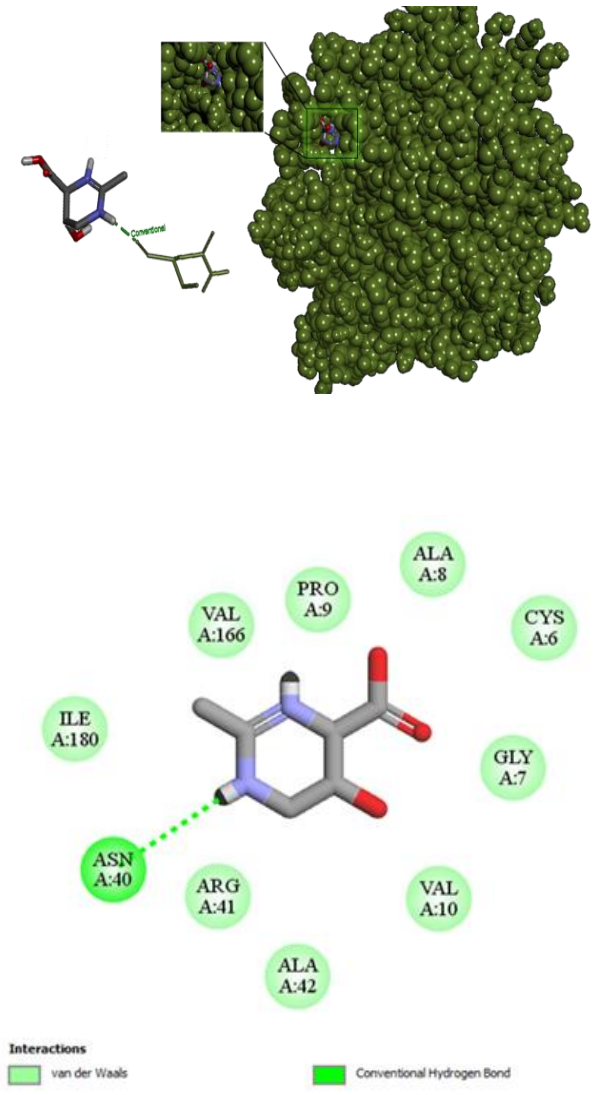
Aeromonas hydrophila ATCC 7966, *Micrococcus luteus* LA 2971, *Mycobacterium smegmatis RUT*, *Bacillus subtilis* IMG 22, *Pseudomonas pyocyanea*, *Bacillus subtilis var. niger* ATCC 10, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 15753, *Bacillus brevis* FMC3, *Klebsiella pneumoniae* FMC 5, *Corynebacterium xerosis* UC 9165, *Bacillus cereus* EU, *Bacillus megaterium* NRS, *Yersinia enterocolytica*, *Listeria monocytogenes* scoot A, *Bacillus megaterium* EU, *Bacillus megaterium* DSM32, *Klebsiella oxytocica*, *Staphylococcus aerogenes*, *Streptococcus faecalis*, *Mycobacterium smegmatis* CCM 2067	The LPO--100 mM thiocyanate--100 mM H_2O_2 system was effective agent against many of the organisms	Bovine LPO	Uğuz and Ozdemir 2005
Brevibacillus centrosaurus, *B. choshinensis*, *B. lyticum*, *Cedecea davisae*, *Chryseobacterium indoltheticum*, *Clavibacter michiganense pv. insidiosum*, *Kocuria erythromyxa*, *K. kristinae*, *K. rosea*, *K. varians*, *Paenibacillus validus*, *Pseudomonas syringae pv. populans*, *Ralstonia pickettii*, *Rhodococcus wratislaviensis*, *Serratia fonticola*, *Streptomyces violaceusniger*, *Vibrio cholerae-nonO1*	LPO system has inhibition effects on all type bacteria	Bovine lactoperoxidase (LPO)	Cankaya et al 2010

6. Docking of hydroxyectoine into the binding site of LPO

The Auto dock tools (ADT) are applied for molecular docking of proteins and enzymes. The crystal structure of LPO from RCSB Protein Data Bank (PDB) is used for further analysis. The three-dimensional structure of hydroxyectoine (as a ligand) is retrieved as the MOL_2 format from ZINC library. Docking of hydroxyectoine into the binding site of LPO has been performed with Auto Dock 4.0 Software. LGA (Lamarckian Genetic Algorithm) has been used to perform a hundred runs of docking. Docking study can predict the exact binding site for hydroxyectoine on the enzyme. The predicted binding models for LPO-hydroxyectoine complex with the lowest docking energy is shown in (Fig. 3a). As shown, there was a single binding site for hydroxyectoine on LPO that confirms the fluorescence spectroscopy outcomes. The 3D structural models of the LPO- hydroxyectoine complex showed that Asn 40 of LPO join the hydrogen bonding interaction with the hydroxyectoine. Moreover, van der Waals interactions also exist (Fig. 3b). The docking results were in coincident with

fluorescence studies that showed static quenching mechanism (Borjian-Boroujeni et al. 2021; Borjian-Boroujeni et al., 2018).

Figure 3: Docking of hydroxyectoine in the binding site of LPO (a), The involved amino acid in formation of H-bond (dash-line) and hydrophobic interaction in LPO-hydroxyectoine complex (b) (Borjian-Boroujeni et al., 2021; 2018).



8. Conclusion

This study elucidated the potential application of lactoperoxidase, a naturally occurring antimicrobial enzyme found in the LPOS, as a viable substitute for chemical preservatives in order to enhance the overall quality, activity and stability of LPO. The antimicrobial effect of LPO is hypothesized to result from the oxidation of sulfhydryl (SH) groups found in microbial proteins and enzymes. This oxidation process involves the formation of intermediate oxidizing products, which subsequently triggers alterations in various cellular functions, including membrane integrity, passage systems, and metabolic enzymes. This system exhibits bacteriostatic and bactericidal capabilities against Gram-positive and Gram-negative microorganisms, consecutively. Moreover, it exhibits noteworthy antifungal and antiviral properties. LPOS-containing films and coatings has exhibited significant antimicrobial activity, leading to improved shelf-life of food products. However, the selection of film types and coatings incorporating LPOS was restricted to alginate, chitosan, gelatin, and whey protein. Ectoine, an osmolyte, affects LPO, finding higher ectoine concentrations improved LPO's activity, binding to LPO influenced its stability and thermodynamics, showcasing ectoine's potential as a stabilizing agent for lactoperoxidase in industrial and medical contexts. Also, the presence of stabilizers appears to enhance the bactericidal efficacy of the enzyme by increasing its enzymatic activity. This observation indicates that the immobilization process offers hope in terms of stabilizing enzymes and managing their inherent instability. Consequently, additional investigations are imperative to assess the antimicrobial attributes of alternative categories of edible films and coatings that encompass LPOS. The results of kinetic studies of LPO can be used for comparative analysis of the properties of lactoperoxidases from different animal species, which will contribute to the development and the introduction of new ways of using these enzymes in the food and pharmaceutical industries as well as for quality control in the production of food and cosmetic products containing LPO. Furthermore, more research is needed to explore the potential effectiveness of the LPOS in

combination with other natural antimicrobial agents and hurdle technology.

Author contribution:

All authors contributed to the study's conception and design. Marziyeh Borjian Boroujeni and Mahmoud Reza Aghamaali, Hashem Nayeri, and Keyvan Beheshti Maal experimented. Marziyeh Borjian Boroujeni wrote the manuscript with support from Mahmoud Reza Aghamaali, Hashem Nayeri, and Keivan Beheshti Maal. Hashem Nayeri and Keyvan Beheshti Maal conceived the original idea. Mahmoud Reza Aghamaali supervised the project.

Conflict of interest

The authors declare no conflict of interest.

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