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Research Article

Probiotic Edible Film Added with Aqueous Clove Extract Milk Powder [1][2]

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Abstract

The integration of aqueous clove extract (ACE) and spray-dried probiotics into an edible film presents numerous health advantages, such as enhanced probiotic stability and antibacterial efficacy against harmful bacteria like *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. This study aimed to formulate a probiotic edible film with antibacterial properties by incorporating milk powder containing ACE and probiotic strains, namely *Streptococcus thermophilus* ATCC 19258, *Lactobacillus bulgaricus* ATCC 11842, and *Lactococcus lactis* MG1363. The milk powders, containing 7.5% (w/v) ACE with various probiotic strains, were combined with 2% (w/v) pectin biopolymer and 60% (w/w) glycerol plasticizer to produce the probiotic edible film with ACE. Antibacterial effects were investigated using disc diffusion, while probiotic film viability was assessed through the pour plate method. The study revealed higher viability in the probiotic strains *L. lactis* and *S. thermophilus* within the edible film, compared to *L. bulgaricus*. The specific strains exhibited increased antibacterial activity against *K. pneumoniae* and *P. aeruginosa*, underscoring the critical role of probiotic strain selection in determining functional properties. Notably, the edible film containing *L. lactis* and *S. thermophilus* demonstrated substantial antibacterial activity against both pathogens. However, further research is imperative to optimize industrial-scale production methods, assess efficacy in diverse food items, evaluate sensory properties, gauge barrier attributes, and investigate the impact of storage conditions on stability and performance.

Keywords: Probiotic edible film, S. thermophilus, L. bulgaricus, L. lactis, Viability, Antibacterial

Introduction

Pneumonia is an acute respiratory infection which affects the lungs, causing cough, nasal congestion, difficulty breathing, syanosis, hypoxia, fever and dizziness [1]. The World Health Organization states that in 2019, an estimate of 740 180 children under 5 years old lost their lives to pneumonia, which accounted for 14% of all deaths [2]. Pneumonia is typically caused by bacterial infection (90%), intoxication, and immuno-deficiency. Opportunistic pathogens *Puedomonas aeruginosa* and *Klebsiella pneumoniae* are known to cause major respiratory tract diseases, gastrointestinal infections, and bacteremia, and are particularly dangerous for those with AIDS, cancer, and severe burns. *K. pneumoniae* and *P. auroginosa* are two of the major pathogens causing pneumonia [3].

Incorporation of fermented products into one's daily diet, along with starter strains of *Lactobacillus bulgaricus* and *Streptococcus thermophils*, help to boost the immune system and can prevent respiratory infections. This is attributed to the immunoregulatory properties of these strains, which in turn strengthens a person's respiratory tract [4]. The study by Fauziah et al.[1] suggested that soyghurt with a concentration of 80% *L. bulgaricus* has the potential to exhibit bactericidal activity against *K. pneumoniae* strains, indicating its potential as a functional food product . Additionally, fermented milk is commonly used as a delivery system for various probiotics and bioactive compounds derived from medicinal herbs, as it can help to improve the viability and stability of probiotics and other bioactive compounds during storage and gastrointestinal transit. It can also provide a convenient and palatable means of delivering these beneficial compounds to consumers [5].

For centuries, clove *(Syzygium aromaticum* L.) has been a popular culinary spice and employed in traditional

medicine to treat different ailments. Clove ingredients have been found to possess antiviral, antibacterial and anti-inflammatory properties, particularly in relation to respiratory issues [6]. The study by Ogwaro et al.[7] reported that the use of clove extracts during fermentation and storage has the potential to improve the microbiological safety of fermented milk. The study found that the addition of clove extracts to fermented milk inhibited the growth of pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhi*, which are known to cause foodborne illnesses. Clove extracts contain natural antimicrobial compounds such as eugenol, which have been shown to exhibit strong antimicrobial activity against various microorganisms. Therefore, the use of clove extracts in fermented milk could provide a natural and effective means of improving its microbiological safety and extending its shelf life.

The starter cultures *S. thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* are commonly used probiotics that have beneficial effects on gut health [8]. *Lactococcus* strains are also important probiotics that have been observed to produce bacteriocins, which are antimicrobial peptides that can inhibit the growth of pathogenic bacteria [9]. The use of edible films for delivering bioactive compounds and probiotics can help to protect these compounds from degradation during storage and transport, increasing their effectiveness and shelf life of probiotics. The incorporation of bioactive compounds and probiotics into edible films has the potential to transform food products into "functional" foods, which can provide additional health benefits beyond basic nutrition. This can add value to the food market and provide consumers with healthier options [10]. The European Food Safety Authority, and Food and Drug Administration have both allotted the group of lactic acid bacteria (LAB) and clove extract the category of "qualified presumption of safety" (QPS) and "generally recognized as safe" (GRAS) respectively, allowing for their use in the food industry [6,11].

Encapsulation is an advanced method for safeguard and increase the shelf life of probiotics, where the active component will be covered by a protective coating made of food-grade material during processing. Spray drying is one among that, spraying probiotic and protective material mixture into a heated chamber, the moisture evaporates and leave dry particles with probiotics [12]. To ensure the survival of probiotics, their insertion in edible films protects them from various physical and chemical hazards during processing and storage [13]. Because of the growing desire for minimally processed foods, edible film created from naturally derived biopolymers become highly popular; this can assist to extend probiotic shelf life and improve quality [14].

Functional probiotic microencapsulation with antibacterial potential is a promising alternative to antibiotics, as it allows the gradual release of functional compounds to prevent diseases [10]. Hence, the present work was aimed to produce fermented milk powder encapsulated in pectin biopolymer with ACE incorporated with *Streptococcus thermophilus* ATCC 19258, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 = JCM 1002, and *Lactococcus lactis* subsp. *tcremoris* MG1363, respectively, their viability in edible film and antibacterial effect against *K. pneumoniae* ATCC 700603 *and P. aeruginosa* ATCC 27853 were determined.

Material and Methods

Study Materials

The research utilized low-fat UHT milk sourced from Nestle products Sdn. Bhd. in Malaysia, *S. thermophilus* ATCC 19258, and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 = JCM 1002, which were isolated from yoghurt by Malaysia Milk Sdn. Bhd. (MariGold, Malaysia). *L. lactis* subsp. *cremoris* MG1363 was obtained from the culture collection at the University of Putra Malaysia, Malaysia. All lactic acid bacteria were confirmed by 16S rRNA sequencing and the data was analysed by BLAST. Pathogenic strains *K. pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853 were obtained from the culture collection of UCSI University.

Preparation of ACE

Clove *(Syzygium aromaticum)* buds were procured from a local market in Cheras, Malaysia (Voucher no. KM 0042/22, Herbarium- Institute of Bioscience, University Putra Malaysia). These buds were oven-dried overnight at 60°C, and then powdered in a mixer and sieved. Subsequently, 10% (w/v) clove powder was added to sterile distilled water and kept at 50°C for 24 h. The resulting ACE was filtrated using a Whatman no. 1 filter paper, followed by a 0.22 µm syringe filter [15].

Preparation of Functional ACE Incorporated Fermented Milks

Fermented milks were prepared with slight modifications of method by Kanik et al.[16]. To produce functional fermented milk samples, 7.5 mL of ACE was added to 92.5 mL of low-fat UHT milk, which was homogenized for 5 min at room temperature. Then the mixture was inoculated with 1% fermented milk starter cultures containing *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, and *L. lactis*. The inoculated fermented milks were then incubated for 18 h at 42°C, 37°C, and 30°C, respectively. The fermented milks were then stored in the refrigerator at 4°C for 24 h before analysis.

Preparation of Functional ACE Incorporated Fermented Milk Powders

For functional fermented milk powder samples production, each fermented milks were spray dried at 4°C feed

temperature, 140°C inlet temperature and 30% pump speed with 100% aspiration (BÜCHI mini spray dryer, B-290, Switzerland) [17]. The fermented milk powder with *L. delbrueckii subsp. bulgaricus, S. thermophilus*, and *L. lactis*, respectively were then stored in the refrigerator (4°C) for 24 h prior to analysis.

Preparation of Functional Edible films with Fermented Milk Powder

Pectin edible film with specific fermented milk powder was prepared according to Ribeiro et al.[18] with slight modifications. The sterile casting solution was formulated using a combination of pectin biopolymer at a concentration of 2% (w/v) and glycerol plasticizer at a proportion of 60% (w/w). For functional edible film samples production, fermented milk powder (7.5%) with *L. delbrueckii subsp. bulgaricus, S. thermophilus*, and *L. lactis*, respectively were added. The inoculated edible films with each fermented milk powders were then dried in oven (Memmert, Germany) for 24 h at 30°C. Prepared edible films were then stored in the dedicator for further analysis.

Viability of Lactic Acid Bacteria

The viable counts of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, and *L. lactis* in samples were determined using the conventional pour plate count technique. *Streptococcus thermophilus* agar (HIMEDIA, India) [19] was used for the enumeration of *S. thermophilus* (37°C for 48 h), modified reinforced clostridial medium (mRCM) [20] for *L. bulgaricus* (37°C for 48 h), and GM17 agar [21] for *L. lactis* (30°C for 48 h). For each sample, 1 g was diluted with 9 mL of normal saline for serial dilution, and the plates were then incubated in an aerobic environment. The results were expressed as colony-forming units per millilitre (log CFU/mL).

Antibacterial Analysis

Mueller Hinton Agar (MHA) was placed on sterilised petri plates and allowed to completely solidify. A bacterial suspension of *K. pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853 (0.1 OD) was equally (100 µL per plate) dispersed onto the surface of the medium using a sterile coating rod [22]. To test the antibacterial activity of fermented milk supernatant, 10 g of fermented milks were centrifuged (MIKRO 220R, Germany) at 4.000 rpm (1431 x g) for 30 minutes at 4°C. The supernatant was then filtered using a 0.22 µm millipore membrane syringe filter before being used [23]. After 10 min in the treatment supernatant, each sterile disc was dried for one minute before being placed on the petri plates. The plates were incubated at 37°C and the diameter of the inhibition zone was measured using a Vernier caliper after 24 h using disc diffusion method [24]. Following that, a reconstituted fermented milk powder solution was made by dissolving 20% (w/v) of the powder in sterile distilled water. Sterile discs were then immersed in the solution for 10 min before being dried for one minute before being tested for antibacterial activity [25,26]. Then disc diffusion method was then used to detect the antibacterial activity of the edible film. The films were clipped into disk-shaped samples with a diameter of 6 mm for analysis [27]. Sterile blank disc and chloramphenicol (10%) were used as the negative and positive controls [28], respectively.

Statistical Analyses

Totally three trials were conducted and the data obtained were subjected to statistical analysis. To find significant differences between the samples ($P<0.05$), all data were subjected to one-way analysis of variance (ANOVA), followed by Tukey's post hoc test with SPSS statistics (IBM SPSS statistics 20 version).

Results

Antibacterial Effect of Aqueous Clove Extract (ACE)

Clove extract is an effective and natural alternative to synthetic preservatives, and is a valuable tool in the food industry for maintaining the safety and quality of food products. Clove has anti-inflammatory, antimicrobial, anti-thrombotic, antioxidant, antimutagenic, and anti-ulcerogenic attributes [29]. The effect of varying concentrations of aqueous clove extract on the antibacterial zone of inhibition (mm) against *K. pneumoniae* and *P. aeruginosa* is presented in *Table 1*.

Viability of Functional Fermented Milks, Fermented Milk Powders and Edible Films

Farmers incorporate different spices into milk either prior to or during fermentation, with the aim of enhancing the flavor and aroma of the fermented product [30]. Moreover, the inclusion of clove in fermented milk aids in its preservation and inhibits the proliferation of bacteria, which can cause food spoilage [7]. Researchers have found that the acceptable concentration of clove in yogurt,

Table 1. *The impact of varying ACE concentrations on the antibacterial zone of inhibition (mm) against K. pneumoniae and P. aeruginosa*

Concentration of ACE (%)	Zone of Inhibition (mm)	
	K. pneumoniae	*P. aeruginosa*
0% (blank)	6.00±0.00^{d}	6.00±0.00^{d}
2.5 %	7.67±0.58^{c}	7.33±0.58^{c}
5 %	9.33±0.58^{b}	9.00±0.00^{b}
7.5 %	11.00±0.00^{a}	10.33±0.58^{a}

Results were expressed as mean ± standard deviation, values are means of triplicates (n=3). ^{abcd} *means in the same columns followed by same uppercase letters are non-significant (P<0.05) via one way ANOVA with tukey test*

Table 2. *The viability and rate of survival of lactic acid bacteria in fermented milk with and without the presence of aqueous clove extract (ACE)*

Fermented Milks	*S. thermophilus*		*L. bulgaricus*		*L. lactis*	
	Viability (log CFU/mL)	Survivability (%)	Viability (log CFU/mL)	Survivability (%)	Viability (log CFU/mL)	Survivability (%)
Without ACE	13.83 ± 0.02^{Aa}	100.00	13.93 ± 0.03^{Aa}	100.00	13.22 ± 0.08^{Ab}	100.00
5% ACE	13.19 ± 0.017^{Ba}	95.35	13.21 ± 0.11^{Ba}	94.83	12.02 ± 0.07^{Bb}	90.92
7.5% ACE	12.21 ± 0.11^{Ca}	88.29	12.10 ± 0.16^{Ca}	86.86	10.78 ± 0.08^{Cb}	81.54

Results were expressed as mean ± standard deviation, values are means of triplicates (n=3). ^{ABC} means in the same column followed by same uppercase letters and ^{ab} means in the same rows followed by same lowercase letters are non-significant (P<0.05) via one way ANOVA with tukey test

Table 3. *Viability and reduction percentage of lactic acid bacteria in ACE-enriched fermented milk, spray dried powder, and edible film*

Sources	*S. thermophilus*		*L. bulgaricus*		*L. lactis*	
	Viability (log CFU/mL)	Reduction (%)	Viability (log CFU/mL)	Reduction (%)	Viability (log CFU/mL)	Reduction (%)
Fermented milk	12.21 ± 0.11^{Aa}	0.00	12.10 ± 0.16^{Aa}	0.00	10.78 ± 0.08^{Ab}	0.00
Spray dried powder	9.74 ± 0.10^{Ba}	20.23	9.39 ± 0.06^{Bb}	22.40	9.75 ± 0.05^{Ba}	9.55
Edible film	8.09 ± 0.06^{Cab}	33.74	7.95 ± 0.05^{Cb}	34.30	8.13 ± 0.08^{Ca}	24.58

Results were expressed as mean ± standard deviation, values are means of triplicates (n=3). ^{ABC} means in the same column followed by different uppercase letters and ^{ab} means in the same rows followed by different lowercase letters are significant (P<0.05) via one way ANOVA with tukey test separately

Table 4. *Antibacterial zone of inhibition (mm) of edible film, spray dried powder and fermented milk against P. aeruginosa and K. pneumoniae*

Source	Zone of Inhibition (mm)					
	S. thermophilus		*L. bulgaricus*		*L. lactis*	
	P. aeruginosa	*K. pneumoniae*	*P. aeruginosa*	*K. pneumoniae*	*P. aeruginosa*	*K. pneumoniae*
Fermented milk	8.67 ± 0.58^{Bb}	8.00 ± 0.00^{Ba}	9.33 ± 0.58^{Aab}	8.00 ± 0.00^{Aa}	10.00 ± 0.00^{Ba}	8.33 ± 0.58^{Ba}
Spray dried powder	9.67 ± 0.58^{ABb}	8.67 ± 0.58^{Ba}	9.33 ± 0.58^{Ab}	8.33 ± 0.58^{Aa}	11.67 ± 0.58^{ABa}	9.67 ± 0.58^{Ba}
Edible film	11.33 ± 1.15^{Aa}	11.00 ± 0.00^{Aa}	8.00 ± 0.00^{Bb}	8.67 ± 0.58^{Ab}	13.33 ± 1.15^{Aa}	11.67 ± 0.58^{Aa}

Results were expressed as mean ± standard deviation, values are means of triplicates (n=3). ^{AB} means in the same columns followed by different uppercase letters and ^{ab} means in the same rows followed by different lowercase letters are significant (P<0.05) via one way ANOVA with tukey test separately for P. aeruginosa and K. pneumoniae

as determined by its sensory attributes, was below the concentration required for microbial inhibition, resulting in a sublethal effect on both *Lactobacillus* sp. and the yogurt starter culture, without compromising the fermentation process. *Table 2* presents the viability and survivability of lactic acid bacteria in fermented milk without ACE and with varying percentages of ACE.

Numerous studies have demonstrated the benefits of microencapsulation in enhancing the survival of probiotics across various manufacturing techniques and unfavourable gastrointestinal conditions. Probiotic products can be sensitive to environmental factors such as temperature, moisture, oxygen, and light. Exposure to unfavorable conditions during processing, packaging, and storage can lead to a reduction in the viability of the probiotic microorganisms, which can, in turn, compromise the efficacy of the product. Edible films made from pectin can improve the quality of food products by acting as a barrier to moisture, oxygen, and other gases that can cause spoilage, resulting in an extended shelf life. Additionally, pectin-based films can provide a protective coating to encapsulate and preserve probiotics [31]. *Table 3* displays the viability of lactic acid bacteria in spray-dried fermented milk powder, and edible film.

Antibacterial Effect of Functional Fermented Milk, Fermented Milk Powders and Edible Films

K. pneumoniae and *P. aeruginosa* are capable of inhabiting the oral cavity, pharynx, and gastrointestinal tract of humans, and are highly virulent and antibiotic resistant. Currently, *K. pneumoniae* is the predominant cause of pneumonia acquired in hospital settings [32]. *Table 4* displays the impact of edible film, spray dried powder, and fermented milk on the antibacterial zone of inhibition (mm) against *P. aeruginosa* and *K. pneumoniae*. In this study, the antibacterial activity of edible films, spray dried powder, and fermented milk containing *S. thermophilus, L. bulgaricus*, and *L. lactis* against *P. aeruginosa* and *K. pneumoniae* was examined. The edible film with *S.*

thermophilus and *L. lactis* shows a significantly higher zone of inhibition against both *P. aeruginosa* and *K. pneumoniae* in the present study, measuring 11.33±1.15 mm and 13.33±1.15 mm, respectively, and 11.00±0.00 mm and 11.67±0.58 mm, respectively. The literature findings reported four categories of active compound inhibition in bacteria, with the diameter of the inhibition zone classified as weak (5 mm), moderate (5-10 mm), strong (10-20 mm), and very strong (20-30 mm) [28].

Discussion

A significant increase in the antibacterial zone of inhibition (mm) against *K. pneumoniae* and *P. aeruginosa*, with values of 11.00±0.00 and 10.33±0.58 respectively, was observed for 7.5% of ACE, in comparison to 5% and 2.5%. In vitro testing was conducted to assess the antibacterial effects of clove ethanolic extract on *K. pneumoniae* and *P. aeruginosa*, resulting in maximum zones of inhibition of 16mm and 14mm, respectively [33]. These results were consistent with previous research by which showed that clove water extract inhibited the growth of *P. aeruginosa*. Furthermore, antibacterial properties were observed in clove extracts prepared from both aqueous and ethanol-based solutions against both *K. pneumoniae* and *P. aeruginosa*, with the most significant activity being observed against *K. pneumoniae* [34].

The viability of lactic acid bacteria in fermented milk was significantly reduced with the addition of ACE, as indicated by the results. The fermented milk without ACE had the highest viability of lactic acid bacteria. These findings suggest that the addition of ACE negatively affected the viability of lactic acid bacteria in fermented milk. The viability of *S. thermophilus*, *L. bulgaricus*, and *L. lactis* fermented milk with 7.5% ACE was observed to be 12.21±0.11, 12.10±0.16, and 10.78±0.08 (log CFU/mL), respectively. Although there is no agreement among the global scientific community regarding the ideal dosage of probiotics needed to obtain health benefits, various studies have suggested that minimum doses ranging from 10^6 to 10^9 CFU/day may be necessary to produce therapeutic effects [11]. For probiotic foods to provide health benefits, they must contain a minimum of 10^7 cfu/g of probiotic bacteria. According to the findings gathered thus far, it can be inferred that fermented milk containing 7.5% ACE integration has demonstrated adequate viability, making it a viable candidate for both spray drying and edible film production, thereby enhancing the longevity of its viability according to Pourjafar et al.[35].

In the present study, it was observed that the viability of *S. thermophilus* and *L. lactis* in fermented milk powder and their respective edible film was significantly higher than that of *L. bulgaricus*. The viable count of edible films prepared with *S. thermophilus*, *L. lactis*, and *L. bulgaricus* in pectin biopolymer were 8.09±0.06 log CFU/mL, 8.13±0.08 log CFU/mL, and 7.95±0.05 log CFU/mL, respectively. Similarly, for spray-dried powder, the viable count was 9.74±0.10 log CFU/mL, 9.75±0.05 log CFU/mL, and 9.39±0.06 log CFU/mL, respectively. These results were consistent with previous reports that *S. thermophilus* was less susceptible to spray-drying than *L. delbrueckii* subsp. *bulgaricus*. In addition, probiotic yogurt powder containing *Lactobacillus paracasei* and *S. thermophilus* had viable counts exceeding 10^8 CFU/g, while the less heat-tolerant *L. delbrueckii* subsp. *bulgaricus* survived only at 10^5 CFU/g of powder. Also the coatings made with *S. thermophilus*, *L. bulgaricus*, and *L. acidophilus* exhibited the best protective properties against microbial spoilage, higher viability (10^8 CFU/g), and increased shelf life [36].

According to Gul & Atalar [37] the viability of *Lactobacillus* sp. reduced by 0.43-1.62 log on spray drying, where *L. paracasei* NFBC 338 and *L. rhamnosus* GG survived spray-drying of reconstituted skim milk at approximately 80% and 60%, respectively. Similarly, the viability of *Lactobacillus* strains after spray-drying process shows 1.69-1.99 log-reduction [38]. Correspondingly, fresh yoghurt *Lactobacillus* viability also dropped from 7.42-6.15 log CFU/g on spray drying, indicating a 17.1% loss in viability [39]. In comparison to different film-forming solutions, edible films containing *Lactobacillus* strains showed a decrease in viability ranging from 0.4 to 2 log CFU/g [40].

Furthermore, Ma et al.[41] reported that the *Lactococcus* strain showed the best viability and antimicrobial activity when added to edible film. A survival rate of 21.6% *for Lactococcus lactis* ssp. *cremoris* was observed to be higher when spray drying was carried out at 130°C, utilizing lactose and sodium caseinate as the drying agents. Various inlet temperatures were used, resulting in a loss of viability ranging from 0.60 to 1.22 log cycles for *Lactococcus* [42]. The study suggested that the reason for the high retention of *Lactococcus* survivability 93.14±2.74%, in edible film, could be linked to the utilization of a lower drying temperature of 30°C and an extended adaptation time of one day [43]. The results were consistent with the present study.

As a result of atypical encapsulation procedure, it is possible to reduce the viability of the *Lactobacillus* and *Lactococcus* strains, which could have consequences for their prospective application of probiotics. The ability of lactic acid bacteria to survive in edible film can be influenced by the base material of the film, nutritional content, initial viability of bacteria, pH, and osmotic pressure. Further research is required to enhance the encapsulation procedure to reduce the loss of viability while maximizing the potential advantages.

Lactococcus has been found to contain antibacterial compounds that inhibit the growth of *Pseudomonas,* making it a potential preventative and treatment option for *Pseudomonas* infections [44]. To ensure the safety of food, researchers have investigated the antimicrobial properties of *Lactococcus* strains found in spontaneously fermented camel milk and the elimination mechanism of *K. pneumoniae* in pasteurized camel milk. All milk samples that were inoculated with a *L. lactis* strain were devoid of *K. pneumoniae* after 75 h of testing [45]. Hence, the antimicrobial activity observed in *L. lactis* cannot be ascribed to the organic acids or hydrogen peroxide generated by the culture, as the inhibitory effects remained unchanged even after neutralization or treatment with catalase in the cell-free supernatant preparations.

P. aeruginosa is an opportunistic pathogen that can cause food spoilage through its lipolytic and proteolytic activities, although this can be prevented by the hydrogen peroxide produced by *Lactobacillus* species. Furthermore, according to Jamalifar et al.[46], *Lactobacillus acidophilus* strain exhibited strong (90 % inhibitory activity) anti-pseudomonal activity against multi-drug resistant clinical isolates. The current findings are in agreement with those of Pulusani et al.[47], *L. bulgaricus* was found to produce a robust zone of inhibition against the growth of *P. aeruginosa*, measuring over 25 mm, while *L. acidophilus* and *S. thermophilus* exhibited zones of inhibition ranging from 21-25 mm. Yerlikaya et al.[48] also reported the highest antimicrobial effect of *L. bulgaricus* (17 ± 1.7mm) than *S. thermophilus* against *P. aeruginosa*.

The current findings align with the outcomes presented by Fauziah et al.[1], signifies that *L. bulgaricus* found in soyghurt can impede the attachment of *K. pneumoniae* to HEp2 cell lines. Following a period of pre-infection, co-infection, and post-infection with *L. bulgaricus* at a concentration of 10^8 CFU/mL for 5 hours, the adhesion of *K. pneumoniae* ATCC 700603 decreased by 6.42%, 19.505%, and 35.405%, respectively. Similarly, the adhesion of *K. pneumoniae* S941 decreased by 10.11%, 37.845%, and 43.74%, while *K. pneumoniae* CT1538 showed a decline of 30%, 31.055%, and 55.875%. In addition, it has been demonstrated that strains of *L. bulgaricus* and *S. thermophilus* isolated from homemade yogurts exhibit antimicrobial activity against *K. Pneumoniae*. Furthermore, the impact of *L. bulgaricus* has been uncovered by researchers who observed that antimicrobial effect was attributed to the unique characteristics of *L. bulgaricus*, which resulted in the production of more advanced lactic acid [49]. Previous studies have demonstrated that lactic acid possesses significant antibacterial and antifungal properties [50].

Pseudomonas aeruginosa and *Klebsiella pneumoniae* are of multidrug-resistant gram-negative bacteria that are frequently associated with bacterial pneumonia. Clove has traditionally been used as a natural cure for a variety of diseases, including respiratory problems, headaches, and sore throats. Current study revealed that combining ACE with specific strains of *S. thermophilus*, *L. bulgaricus*, and *L. lactis* in fermented milk, spray dried powder, and edible films can potentially have an antibacterial impact against *P. aeruginosa* and *K. pneumoniae*. The recommended lactic acid bacteria viability for the final edible film made with these strains was 10^7 CFU/mL. *L. lactis* and *S. thermophilus* ACE integrated edible film and spray dried powder were found to have much higher viability and antibacterial action than *L. bulgaricus*. Lactic acid bacteria (LAB) are known to produce organic acids that reduce pH and enhance the formation of hydrogen peroxide and bacteriocins, both of which have antibacterial effects against a variety of pathogenic pathogens, including Gram-positive and Gram-negative bacteria. However, further research is needed, to fully understand the combined effects of multiple natural antimicrobial agents and lactic acid bacteria, its mechanisum of action in an actual food system to establish its potential value in preventing foodborne diseases.

Declarations

Availability of Data and Materials: The datasets used and/or analysed during the current study are available from the corresponding author (L. P. Pui) on reasonable request.

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Authors Contribution: Study design: LAKS and LPP; Experiments: LAKS; Data analysis: LAKS, LPP and KLN; Writing - Original draft preparation: LAKS; Writing - Review & Editing: all authors; All authors read and approved the final manuscript.

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