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ANTIBACTERIAL ACTIVITY AND ORGANIC ACIDS FORMATION BY *LACTOBACILLUS* SP. ORIGINATED FROM PICKLED GUAVA AND PAPAYA

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Abstract: Lactic acid bacteria (LAB) produce several antibacterial compounds, including organic acids that inhibit many types of pathogenic bacteria. The antibacterial activity of LAB with the ability to inhibit growth of pathogenic bacteria associated with foodborne illness is seen as a natural way to improve food safety. This study was carried out to isolate and identify LAB from local pickled guava (*Psidium guajava*) and papaya (*Carica papaya*) and to evaluate their antibacterial activity against selected foodborne pathogens. Standard method was used for the isolation of LAB, while identification was done based on their morphological characteristics, biochemical reaction and polymerase chain reaction (PCR) amplification of 16S rRNA gene and sequencing. This study evaluated the ability of cell free supernatant (CFS) of the identified LAB to inhibit the growth of selected Gram-positive and Gram-negative foodborne pathogens through microtiter plate method. Determination of the organic acids formation in the CFS that are responsible for the antibacterial activity of the LAB was also conducted using high-performance liquid chromatography (HPLC). The results showed that three LAB from the genus *Lactobacillus* have been successfully isolated and identified as *Lactobacillus plantarum* (LABP), *Lactobacillus reuteri* (LABR) and *Lactobacillus paracasei* (LABC). All three *Lactobacillus* sp. were able to demonstrate antibacterial activity against foodborne bacterial pathogens used in this study. The results also suggested that the antibacterial activity of CFS of all three *Lactobacillus* sp. was due to organic acids production.

Keywords: Lactic acid bacteria, cell free supernatant, antibacterial, pickled fruits, foodborne pathogen.

Introduction

Foodborne diseases cause by pathogenic bacteria are a major public health problem and remain a relevant issue throughout the world. Millions of people fall ill every year resulting from eating unsafe food contaminated with pathogenic bacteria, such as *Escherichia coli* and *Salmonella* sp. Application of antibacterial agent in the form of natural food preservatives is seen as having a huge potential to prevent or control the growth of pathogenic bacteria in the food industry (Thielmann, 2017). The application of antibacterial agents from natural sources will provide an alternative to synthetic chemical antibacterial agents widely used in industry that has been associated with long-term detrimental effects towards human health (Nuryana, 2019). LABs are generally recognised as safe (GRAS) by the United States Food and Drug Administration (USFDA), indicating it as safe for human consumption (George *et al*., 2018). Other than that, 50 of the recognised LAB members also have the qualified presumption of safety (QPS) status granted by the European Food Safety Agency where most of the LAB are from the *Lactobacillus* sp. (Ricci *et al*., 2017).

LAB are widely spread and can be found in many environments such as soil, plants, raw food, fermented food and the mucosal surfaces of human and animals. The various pressures and conditions in each environment are key factors for the LAB genomic diversity and variation (McAuliffe, 2018). Thus, isolation work of LAB from various and novel sources is an important

part in LAB research and must be done based on their intrinsic characteristics, intended use and future application. In recent years, researchers have reported on the LAB species with antibacterial property isolated from pickled vegetables and fruits. However, it was found that there was more LAB isolation work performed on pickled vegetables compared to pickled fruits, including among others, chili (Shahidah *et al*., 2016), cabbage, bitter bean, garlic and radish (Sukirah *et al*., 2017). LAB isolation studies on pickled fruits included jackfruit, plum, lemon, olive, apple and dates (Roy & Rai, 2017). Thus, this study was carried out to isolate and identify LAB from local pickled guava and papaya and also to evaluate their antibacterial activity using cell free supernatant (CFS) against commonly known foodborne pathogens.

Materials and Methods

Isolation of Lactic Acid Bacteria (LAB) from Local Pickled Guava and Papaya Samples

Pickle samples were collected randomly from a variety of sources, including supermarkets and wet markets in Hulu Langat, Selangor, Malaysia. Samples then were taken to laboratory for analysis. 10 grams of each sample were obtained using aseptic technique and was homogenised in a stomacher (Seward Medical, UK) for 30 seconds in 90 mL of Ringers solution (Oxoid, UK). Serial dilution was performed using 1 mL of the prepared homogenate into 9 mL sterile Ringers solution and three consecutive dilutions that could provide single colony growth were chosen. An amount of 1 mL aliquots of each dilution was transferred to a Petri dish using the pour plate method. The sterile warm (45°C ± 2°C) molten deMan, Rogosa and Sharpe agar (MRS, Difco, USA) of approximately 15 mL was poured into plates to form a layer, swirled and left to solidify. The solidified plates were incubated anaerobically at 37°C ± 2°C for 24 to 48 hours.

After incubation period, each plate was examined and those containing single, white or cream round shape colonies were selected for sub-culturing onto fresh MRS agar following the method done by Kam *et al*. (2011). The sub-culturing process was done three times and the isolates that maintained their characteristics during each sub-culture were grown in MRS broth (Difco, USA) and supplemented with 20% glycerol (Sigma, USA) before being stored at -20°C ± 2°C until being used for further analysis and study.

Identification of LAB Isolates Through Biochemical and Molecular Methods

Identification of the isolated LAB was preliminary done using biochemical identification involving Gram staining and catalase reaction tests. The Gram staining was conducted following the standard protocol for Gram staining while catalase reaction was performed on sterile microscope glass slide using 3% hydrogen peroxide (Sigma, USA) according to Astuti (2016). The Gram staining result was recorded as to whether the isolates were of Gram-positive or Gram-negative bacteria, while the catalase reaction was recorded as either positive or negative of catalase activity.

The isolates having Gram-positive and catalase negative reaction were further identified using molecular method through amplification and sequencing of 16S rRNA gene following the method used by Nur ilida *et al*. (2018). The set of primers used were 27-f : 5'- AGT TTG ATC CTG GCT CAG -3' and 1492-r : 5'- GTT TAC CTT GTT ACG ACT T-3'. The QIAamp® DNA Mini Kit (QIAGEN, USA) was used to extract genomic DNA of each isolate and the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using a thermal cycler in a total volume of 50 µL under the following conditions: 1 cycle of 95°C for 15 seconds; 40 cycles of 95°C for 15 seconds, 55°C for 15 seconds and 72°C for 30 seconds; and 1 cycle of 72°C for 10 minutes. The PCR products were then separated on 1% (w/v) agarose gel (GeneDirex, Taiwan) containing GelRed (Biotium, USA) using electrophoresis with constant voltage of 80 V for 55 minutes in 1 x TBE buffer (UltraPure, USA). The sizes of DNA fragments were estimated using 1000 base pairs (bp) DNA ladder. The PCR products were submitted to First Base Laboratories Sdn Bhd

for sequencing and the nucleotide sequences obtained were later analysed using the BLAST program available online at *https://blast.ncbi.nlm.nih.gov/Blast.cgi*.

Preparation of LAB Isolates and Pathogenic Strains for Antibacterial Activity Study

The identified LAB isolates then were subjected to antibacterial activity study and the preparation of the LAB culture was done according to Arena *et al*. (2016). All of the LAB isolates which were previously maintained at -20°C ± 2°C in MRS broth supplemented with 20% glycerol a stock were taken. An amount of 0.1 mL of the LAB glycerol stock were inoculated in 5 mL MRS broth, tightly screwed cap and incubated at 37°C ± 2°C for 24 hours. Later, another 1 mL was taken from the overnight culture and transferred into 10 mL MRS broth and incubated for 24 hours prior to antibacterial study.

The pathogenic strains were also previously stored in glycerol at -20°C ± 2°C and were revived prior to use by transferring 20 µL of the glycerol stock into 5 mL Tryptic soy broth (TSB, Oxoid, UK) and incubated at 37°C ± 2°C for 24 hours. Later, 1 mL of these cultures were transferred into 10 mL TSB broth and again incubated at 37°C ± 2°C for 24 hours. The bacterial suspension was then prepared to a turbidity of 0.5 McFarland standard (~1 x10^8 colony forming units CFU mL^{-1}) by adding the sterile distilled water into the suspension until the desired turbidity was achieved. The desired turbidity was determined by using McFarland nephelometer (Becton Dickinson, USA). The pathogenic bacteria used were Gram-positive *Listeria monocytogenes* ATCC® 7644™ and two Gram-negative organisms, *E. coli* ATCC® 48888™ and *Salmonella enterica* serovar Typhimurium ATCC® 14028™.

Antibacterial Activity Study of LAB Cell Free Supernatant (CFS) Using Microtiter Plate Method

The antibacterial activity of the LAB CFS was conducted following the method used by Hor & Liong (2014). A 10 mL of overnight culture of each LAB was centrifuged at 10,000 rpm for 10 minutes at 4°C. The pellet was discarded and the CFS was filter-sterilized through a sterile 0.45 µm pore size filter (Sartorius Stedim, France). The pH of CFS for each of the LAB was evaluated using a pH meter (Eutech, Singapore). The CFS was then divided into four portions prior to antibacterial activity determination. The first portion (i) was the original CFS identified as untreated CFS (pH 4.07 to pH 4.45). The second and following portions of the CFS were treated as follows: (ii) CFS was adjusted from their initial pH (pH 4.07 to pH 4.45) to pH 6.00 ± 0.20 using sterilized 1 N NaOH and filtered through sterile 0.45 µm pore size filter (Sartorius Stedim, France); (iii) CFS was treated with 1 mg mL^{-1} of catalase (Sigma-Aldrich, USA) at 25°C ± 2°C for 30 minutes and (iv) 1 mg mL^{-1} of proteolytic enzyme, trypsin (Sigma-Aldrich, USA) at 37°C ± 2°C for 2 hours. An amount of 100 µl of the untreated CFS, neutralised CFS and enzymes treated CFS (catalase and trypsin) was pipetted into each well of the microtiter plate. Later, 100 µl of prepared pathogen suspension was pipetted and mixed with each of the different CFS in the well. Another well for control was used, where 100 µl of the pathogen suspension was mixed with 100 µl of MRS broth to replace untreated CFS and treated CFS. This experiment was performed using bacterial strains as mentioned in the above section. All microtiter plates were incubated at 37°C ± 2°C for 48 hours in an incubator. Bacterial growth of the pathogen after incubation was recorded as optical density (OD) reading and the reading was taken using VersaMax™ ELISA Microplate Reader (Molecular Devices, USA). The percentage of growth inhibition was calculated using the below equation:

OD value (control) - OD value (sample)/OD value (control) x 100

The value of OD for control was taken from the well containing pathogen with MRS broth while the OD for sample was taken from the well with pathogen in untreated CFS or treated CFS.

Statistical Analysis

Data were analyzed using SAS 9.3 statistical software (SAS Institute Inc., USA). A one-way analysis of variance was performed to evaluate significant differences between sample means. The level of significance was set at $\alpha = 0.05$. All experimental results were expressed as mean values obtained from three replicates ($n = 3$) unless stated otherwise.

Results and Discussion

Biochemical and Molecular Identification of Isolated LAB

The colony morphology on MRS agar for all isolates (coded as LABP, LABR and LABC) were round in shape and white in color and met the LAB characteristic on MRS agar as described by Astuti (2016). The isolates were all Gram-positive bacteria in rod form, as observed during Gram staining, and they did not produce bubbles in the catalase reaction test, thus identified as catalase negative. Both tests (the Gram staining and catalase reaction) are the most common test used for preliminary identification of LAB based on biochemical reactions. The Gram staining is to identify bacteria based on cell wall characteristics and the LAB are known as Gram-positive bacteria that have a peptidoglycan-rich cell wall. The LAB are also generally known as catalase negative where they produce small amount of catalase enzyme thus are not able to break hydrogen peroxide into water and oxygen (Astuti, 2016).

The isolates then were identified as *Lactobacillus plantarum* (LABP), *Lactobacillus reuteri* (LABR) and *Lactobacillus paracasei* (LABC) through PCR amplification of 16S rRNA gene and sequencing. The sequencing results were compared for similarity with reference species of bacteria contained in genomic database bank using BLAST algorithm and the identification results are as shown in Table 1.

Lactobacillus sp. was a common LAB species successfully isolated from pickled fruits and vegetables as also discovered by other researchers. Kumari *et al*. (2018) isolated seven isolates of LAB from pickled wild Himalayan fig. Isolation of LAB from fermented vegetables done by Kazemipoor *et al*. (2012) have identified four types of *Lactobacillus* sp. from their samples which were *L. animalis*, *L. rhamnosus*, *L. fermentum* and *L. reuteri*. The species *L. reuteri* was also isolated in this study from the pickled papaya. Other than *Lactobacillus* sp., other researchers have also isolated other types of LAB genus from their pickled vegetables and fruits samples such as *Pediococcus* sp. (Roy & Rai, 2017) and *Leuconostoc* sp. (Nur ilida *et al*., 2018).

Table 1: LAB isolates identification by sequencing of 16S rRNA gene

Sample	Code of LAB isolates	Best match in BLAST analysis	Accession number of the best match in BLAST analysis	Identity score (% similarity)
Pickled guava	LABP	*Lactobacillus plantarum*	NR_104573.1	95%
Pickled papaya	LABR	*L. reuteri*	NR_113820.1	97%
Pickled papaya	LABC	*L. paracasei*	NR_117987.1	97%

Antibacterial Activity of Identified LAB Cell Free Supernatant (CFS)

The antibacterial study has shown that the untreated CFS of LABP, LABR and LABC having the ability to inhibit the growth of pathogenic species used in this study which were *L. monocytogenes* ATCC® 7644™, *E. coli* ATCC® 48888™ and *S. enterica* serovar Typhimurium ATCC® 14028™ (Table 2). The results also showed that the inhibition was at different range of percentage depending on both CFS LAB isolates (LABP, LABR or LABC) and pathogen strains used.

The LAB produced various inhibitory compounds that are responsible for its antibacterial activity usually the organic acids, hydrogen peroxide, bacteriocin and these compounds were produced during their growth and excreted extracellularly (Ozcelik *et al*., 2016). Thus, the antibacterial activity evaluation in this study was designed to also able to determine the inhibitory compound that might contribute most to the antibacterial activity of the LAB other than basic screening of their CFS antibacterial performance against pathogens used. For that purpose, the antibacterial activity of LABP, LABR and LABC was evaluated using a few different portions of CFS, namely untreated CFS (original prepared CFS without any treatment and maintain their original condition), neutralized CFS, catalase treated CFS and trypsin treated CFS. It was found that the neutralization of the CFS of all LAB isolates showed very low growth inhibition if compared to the growth percentage caused by the untreated CFS and also catalase and trypsin treated CFS (Figure 1, Figure 2 and Figure 3).

The results indicate that untreated CFS with acidic condition due to the presence of organic acid might have contributed to the antibacterial

Table 2: Antibacterial ability of untreated CFS of LABP, LABR and LABC against selected pathogens

Code of LAB Isolates	Pathogen Growth Inhibition (%)		
	***Listeria monocytogenes* ATCC® 7644™**	***Escherichia coli* ATCC® 48888™**	***Salmonella enterica* Serovar Typhimurium ATCC® 14028™**
LABP	75.63±0.44	89.68±2.32	89.43±1.75
LABR	67.32± 2.54	86.67±0.19	80.46±0.24
LABC	76.30± 2.71	85.31±0.65	85.08±1.26

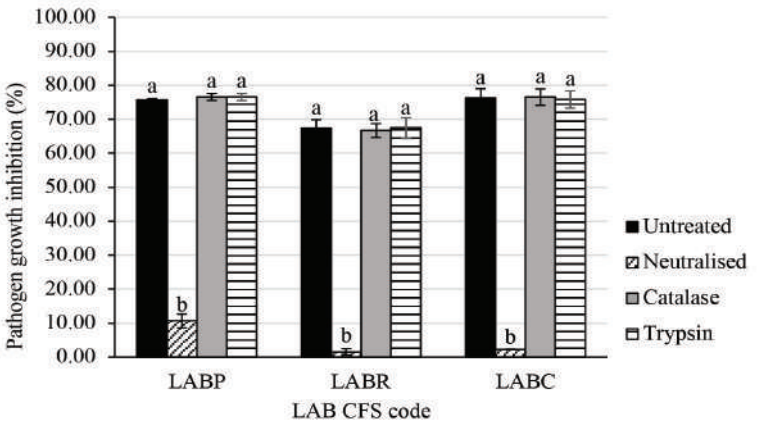

Figure 1: Antibacterial effect of untreated CFS, neutralized CFS, catalase and trypsin treated CFS on the pathogen growth inhibition of *Listeria monocytogenes* ATCC® 7644™. Error bars represent standard error of means (n=3). [a-b]Different lowercase letters indicate that the percentage within the same LAB CFS code are significantly different ($p<0.05$)

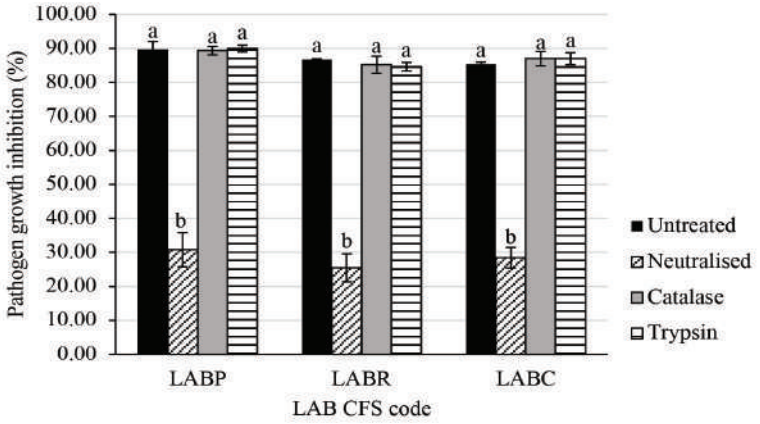

Figure 2: Antibacterial effect of untreated CFS, neutralized CFS, catalase and trypsin treated CFS on the pathogen growth inhibition of *Escherichia coli* ATCC® 48888™. Error bars represent standard error of means (n=3). [a-b]Different lowercase letters indicate that the percentage within the same LAB CFS code are significantly different ($p<0.05$)

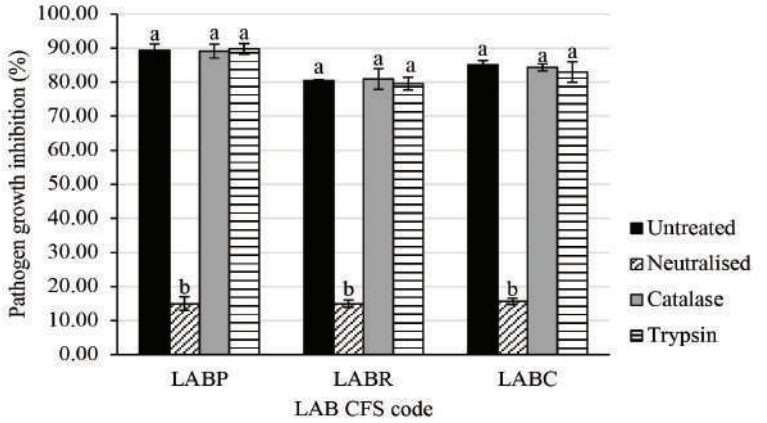

Figure 3: Antibacterial effect of untreated CFS, neutralized CFS, catalase and trypsin treated CFS on the pathogen growth inhibition of *Salmonella enterica* serovar Typhimurium ATCC® 14028™. Error bars represent standard error of means (n=3). [a-b]Different lowercase letters indicate that the percentage within the same LAB CFS code are significantly different ($p<0.05$)

activity of the isolates as also mentioned by Hor & Liong (2014). The LAB produced organic acids naturally as a major fermentation product of their metabolite pathways (Nuryana, 2019). Enzyme treatment on CFS using catalase and trypsin have no significant effect to the antibacterial activity of all LAB isolates against all pathogens if compared to untreated CFS. This was witnessed by the growth inhibition percentage of all pathogens were almost similar when in contact with untreated CFS and also with catalase and trypsin treated CFS. The catalase treatment was performed to rule out the antibacterial activity of CFS was due to hydrogen peroxide. The catalase enzyme will break hydrogen peroxide compound into water and oxygen and reduce the hydrogen peroxide amount in the CFS (Ozcelik *et al*., 2016). The ability of catalase treated CFS with reduced amount of hydrogen peroxide to maintain their inhibitory effect against pathogens confirms that hydrogen peroxide might not contribute to the

antibacterial activity of the strains. It has been recorded that LAB also were able to produce hydrogen peroxide that can inhibit pathogenic bacteria through superoxide anion chain reaction that will produce toxic oxidation (Vieco-Saiz *et al*., 2019). Inhibition action by hydrogen peroxide however, depends a lot on environmental factors, such as pH and temperature. *Lactobacillus* sp. namely *L. johnsonii* and commensal vaginal LAB, such as *L. crispatus*, *L. jensenii* and *L. gasseri* are among commonly known hydrogen peroxide producing species (Hertzberger *et al*., 2014). *Enterococcus faecium* and *Lactococcus lactis* were also among LAB strains reported as able to produce hydrogen peroxide with strong antibacterial effects against pathogens (Surendran *et al*., 2017).

Other than organic acids and hydrogen peroxide, proteinaceous compounds, such as bacteriocin, might be present, or at least in very low amount, in the CFS as an antibacterial compound. Proteolytic enzymes, including trypsin, will inactivate the proteinaceous compound when added to the CFS (Vieco-Saiz *et al*., 2019). The ability of the trypsin treated CFS in this study to maintain their inhibitory effect after inactivation of proteinaceous compound when compared to untreated CFS shows that the proteinaceous compound might not be responsible for the antibacterial activity of the CFS. In a study conducted by Burgenstock *et al*. (2020), they found that 10 out of 21 LAB isolated from traditionally processed sausages have lost their antibacterial activity against selected pathogens after neutralising of the CFS. Another 11 LAB isolates that were not affected by neutralisation however, lost their antibacterial activity after CFS enzymatic treatment with trypsin. They have concluded that the antibacterial activity of the 11 LAB isolates was due to bacteriocins.

Organic Acids Detection in LAB Cell Free Supernatant (CFS)

The type of organic acids formed by each LAB in MRS broth in this study was also evaluated using HPLC and the results are summarised in Figure 4. Several types of organic acids were detected, which were lactic acid, acetic acid, citric acid, tartaric acid and succinic acid. The result in this study shows that all three LAB isolates produced lactic acid at sginificantlt higher concentrations if compared to acetic acid, citric acid, tartaric acid and succinic acid ($p<0.05$). LAB produced many types of organic acids as end products during growth and fermentation. These organic acids provide an acid environment to the medium and become unfavorable for the growth of other types of microorganisms, including pathogenic and spoilage microorganisms (Teusink & Molenaar, 2017).

Several types of organic acids usually produced by LAB and the type and amount secreted varies depending on several factors, including LAB strains and their growth substrate (Nuryana *et al*., 2019). Hor and Liong (2014), found that the LAB strains in their study produced higher concentration of lactic acid than acetic acid. Lactic acid usually produced by LAB as a result of carbohydrate catabolism through either homo or hetero-fermentative pathways (Mora-Villalobos *et al*., 2020) and many LAB strains were found to usually produce more than 250 mg/L in MRS broth (Ozcelik *et al*., 2016). The *L. plantarum* in MRS broth produced concentration of lactic acid of 509.40 mg/L while *L. acidophilus* produced 515.09 mg/L in a study done by Ozcelik *et al*. (2016). In another study, the *L. plantarum* produced lactic acid concentration of up to 6080 mg/L in MRS broth (Vodnar *et al*., 2010). Poppi *et al*. 2015 reported that several LAB species in their study produced almost same amount of lactic acid in MRS broth where *L. plantarum* produced up to 15 000 mg/L of lactic acid, while *L. reuteri* (16 100mg/L) and *L. casei* (8600 mg/L). Thu *et al*. (2013) and Oulkheir *et al*. (2015) also found the ability of LAB strains to produce several types of organic acids, while Bae and Lee (2015) said that the differences in concentration of each organic acid produced in the growth media depended on the LAB strain and each acid will also cause different antibacterial activity.

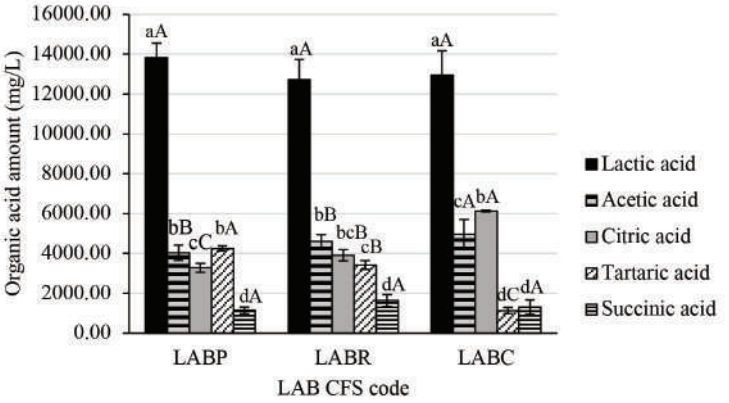

Figure 4: Organic acid formation (mg/L) by LAB in MRS broth. Error bars represent standard error of means (n=3). [a-d]Different lowercase letters indicate that the different organic acid type amount within the same LAB code are significantly different ($p<0.05$). [A-C]Different capital letters indicate that the same organic acid type amount between the different LAB CFS code are significantly different ($p<0.05$)

Conclusion

Three LAB isolates identified as *L. plantarum* (LABP), *L. reuteri* (LABR) and *L. paracasei* (LABC) were isolated from pickled guava and papaya samples. The antibacterial activity study shows that all isolates have inhibitory growth effect against *L. monocytogenes* ATCC® 7644™, *E. coli* ATCC® 48888™ and *S. enterica* serovar Typhimurium ATCC® 14028™ and the effect was due to organic acid. This finding suggested that these LAB isolates may have potential to be used as natural food preservatives. The study also provided a basis for further research regarding the antibacterial component of the isolated LAB and methods for their application in the food industry as an antibacterial agent.

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ARTICLES FOR FACULTY MEMBERS

EFFECT OF LACTIC ACID BACTERIA AS BIO-PRESERVATION AGAINST SPOILAGE FUNGI FROM PAPAYA FRUIT

Antifungal activity and mode of action of lactic acid bacteria isolated from kefir against Penicillium expansum / Chen, H., Ju, H., Wang, Y., Du, G., Yan, X., Cui, Y., Yuan, Y., & Yue, T.

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Antifungal activity and mode of action of lactic acid bacteria isolated from kefir against *Penicillium expansum*

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ABSTRACT

Penicillium expansum is the most common spoilage-associated organism found in fruit, which causes serious economic loss and public health concerns. The aim of this study was to isolate lactic acid bacteria (LAB) from kefir in order to study its antifungal effect on mold growth and further analyze the possible antifungal mode of action of LAB, including antifungal component, lytic enzymes activity and nutrient competition. *Lactobacillus kefiri* M4 and *Pediococcus acidilactici* MRS-7 were isolated from kefir, and the overlay method was used as a qualitative approach to evaluate their antifungal activities against *Penicillium expansum*, the results showed that all tested strains were inhibited by the isolates, except for *P. expansum* LPH6. Different treatments were carried out to characterize the nature of the molecules that contributed to the antifungal activity of the cell-free supernatants produced by the isolates, and it was shown that all supernatants were pH-dependent, partly heat sensitive, and were not influenced by proteinaceous treatment. The cell-free supernatants of both species of LAB were analyzed by high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS), and it was found that the main antifungal compounds were organic acids and carboxylic acids. Both LAB species were able to produce lytic enzymes, such as cellulases, proteases, and glucanases, which formed a halo zone on the plate. L. kefiri M4 and *P. expansum* LPH10 exhibited nutrient competition when co-cultured together in a tissue culture plate insert. The results of this study showed that *L. kefiri* M4 and *P. acidilactici* MRS-7 isolated from kefir possess antifungal activity, which provides useful information of how *P. expansum* response to the LAB isolated from kefir.

1. Introduction

Penicillium expansum, the most important plant pathogen in harvested fruits and vegetables, causes blue mold disease in harvested apples, pears, peaches, and other fruit (Julca, Droby, Sela, Marcet-Houben, & Gabaldon, 2016; Santos, Abrunhosa, Venancio, & Lima, 2002), which results in significant economic losses. Patulin (PAT), a secondary metabolite of *P. expansum*, is ubiquitous in fruit and fruit derivatives (Spadaro, Ciavorella, Frati, Garibaldi, & Gullino, 2007; Spadaro, Garibaldi, & Gullino, 2008). Since PAT has been shown to exhibit carcinogenic, teratogenic and mutagenic effects (Puel, Galtier, & Oswald, 2010; Spadaro, Lore, Garibaldi, & Gullino, 2013), it may cause acute and chronic toxicity to the human body. Therefore, inhibition of *P. expansum* growth and PAT production is of great importance.

Various methods have been applied to prevent fungal mycelial growth, inhibit fungal spore germination, and block toxin production, such as chemical, physical, and biological methods, as well as the synergistic combination of methods. Chemical treatment, such as chitosan (Wang, Wu, Qin, & Meng, 2014), exogenous potassium phosphite (Lai et al., 2017), chlorine dioxide (Zhang & Fu, 2018), and natamycin (He, Zhang, Li, Xu, & Tian, 2019), have a good antifungal effect but its side effects on health and environment limit its development. Physical methods, such as pulsed-light and low-pressure mercury lamp treatment (de Souza, Popovic, Warriner, & Koutchma, 2020), lead to the loss of nutrition in fruit (Aron Maftei, Ramos-Villarroel, Nicolau, Martin-Belloso, & Soliva-Fortuny, 2014). Thus, there is a great demand

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to develop safe and effective antifungal control methods to improve or replace the current chemical and physical treatments.

The use of biological methods to control *P. expansum* has been reported. Research regarding biological control agents mainly focused on yeasts and lactic acid bacteria (LAB) (Ngolong Ngea et al., 2020; Sadiq et al., 2019; Siedler, Balti, & Neves, 2019). Rodriguez Assaf et al. (2020) isolated several fungi, including *Aureobasidium pullulans*, *Cryptococcus magnus*, *Metschnikowia pulcherrima,* and *Rhodotorula glutinis,* with antifungal activity from fermentation microenvironments and the surface of refrigerated grapes (Rodriguez Assaf et al., 2020). Zhang, Spadaro, Garibaldi, and Gullino (2010) reported that several yeasts, which belong to the genus *Metschnikowia,* were able to effectively control blue mold rot (Zhang et al., 2010). Ruggirello et al. (2019) tested the antifungal activity of LAB isolated from cocoa bean fermentation against spoilage fungi belonging to the *Aspergillus* and *Penicillium* genera (Ruggirello et al., 2019). *L. plantarum* K35 displayed potent antifungal activity against the aflatoxin-producing strains of *A. flavus* TISTR304 and *A. parasiticus* TISTR3276 (Sangmanee & Hongpattarakere, 2014). *L. casei* AST18, which was isolated from traditional Chinese dairy products, inhibited the growth of *P. chrysogenum* (Li et al., 2014).

LAB have antifungal properties, such as the production of antifungal compounds, the activity of lytic enzymes, and competitive growth (Bianchini & Bullerman, 2009). During LAB fermentation, the production of organic acids, including lactic acid, acetic acid, formic, propionic, butyric, and others, lowers the environmental pH and causes intracellular acidic stress, resulting in unfavorable conditions for the growth of fungus in food products (Ammor, Tauveron, Dufour, & Chevallier, 2006). Lytic enzymes produced by bacteria can degrade the cell wall of microorganisms and have been demonstrated as a biocontrol mechanism of plant diseases (Kaur, Munshi, Singh, & Koch, 2010). In addition, the competition for nutrients, as seen between *A. pullulans* and *P. expansum* (Janisiewicz, Tworkoski, & Sharer, 2000) and *S. plymuthica* and *P. digitatum* (Meziane et al., 2006), has also been investigated as a biocontrol mechanism. Apart from LAB, kefir and kefir grains have been reported for their antifungal activities (Cevikbas et al., 2010). Iraporda et al. (2017) revealed that the inhibitory activity of kefir on pathogenic microorganisms may be related to a complex microbiota and microbial metabolites (Iraporda et al., 2017). However, there are few studies regarding the LAB isolated from kefir to control *P. expansum* growth.

This study aimed to identify and investigate the antifungal activity of LAB isolated from kefir. The inhibitory effect of the isolated LAB against the growth of *Penicillium expansum* was determined. We characterized the antifungal compounds and used biochemical methods to qualitatively and quantitatively analyze the antifungal metabolites in the active cell-free culture supernatants (CFCSs) of the LAB. The ability of *L. kefiri* M4 and *P. acidilactici* MRS-7 to produce lytic enzymes was evaluated. Finally, nutrient competition between *L. kefiri* M4 with *P. expansum* LPH10 was assessed in order.

2. Material and methods

2.1. Plant pathogens and apple juice

P. expansum LPH6, LPH9, LPH10, F-WY-12-02 were obtained from apple and kiwifruit isolation in our laboratory (Wang et al., 2017; Wang, Yuan, Liu, Zhang, & Yue, 2016). *P. expansum* CGMCC 3.3703 was obtained from the China General Microbiological Culture Collection Center. All pathogens were maintained on potato dextrose agar (PDA) medium (Haibo, Qingdao, China) at 4 °C. Spore suspensions were harvested by removing the spores from a 7-day-old culture on PDA and suspending in sterile distilled water. Spore concentrations were adjusted to an appropriate concentration (10^4 spores/mL) with a hemocytometer. Apple juice (14 °Brix) was prepared by previous method (Wei, Zhang, Yuan, Dai, & Yue, 2019), then was diluted with sterile water to make 1%, 5%, 10% (v/v) apple juice for research.

2.2. Isolation of LAB from kefir

Kefir grains were obtained from a private household (Anhui, China). Kefir was prepared by adding kefir grains to sterilized milk at a proportion of 5% w/v, which was then fermented for 24 h at 25 °C. A loop of kefir milk was streaked onto Man, Rogosa and Sharpe (MRS) agar (Haibo, Qingdao, China) and incubated anaerobically at 30 °C for 72–96 h. Colonies were subcultured twice on MRS agar under anaerobic conditions. Genomic DNA extraction was performed by using the TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China) following the manufacturer's protocol. The sequencing and identification of the isolates were determined as previously described (Jeong et al., 2017). PCR products were sequenced using the primer pair 27F/1492R. A sequence comparison of each isolate was completed using the National Centre of Biotechnology Information (NCBI) database. The identities of the isolates were determined on the basis of the highest Basic Local Alignment Search Tool (BLAST) score. A neighbor-joining phylogenetic tree of *L. kefiri* M4 and *P. acidilactici* MRS-7 was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) Software Version 7.0.

2.3. In vitro assays

In order to test the antagonistic capability of *L. kefiri* M4 and *P. acidilactici* MRS-7, the overlay method was used as previously described with some modifications (Magnusson & Schnurer, 2001). First, a loop of each isolate was propagated twice at 37 °C for 24 h in MRS broth. Then the liquid culture was streaked onto MRS agar plates in two lines (3 cm in length) and incubated at 37 °C for 24 h. Each plate was then overlaid with 10 mL of soft PDA (0.8% agar) containing 10^4 spores/mL of *P. expansum*. All plates were incubated at 28 °C. Fungal growth was examined after 7 days of incubation. The experiment assaying inhibitory activity was performed in duplicate and the antifungal activity was expressed as "strong inhibition (+++), medium inhibition (++), weak inhibition (+), no inhibition (−)" (Fig. S1).

2.4. Characterization of the antifungal activities of CFCSs

L. kefiri M4 and *P. acidilactici* MRS-7 were propagated twice at 37 °C for 24 h in MRS broth. Then the bacterial cells were centrifuged at 10,000 rpm for 10 min, and the supernatant was collected and filtered with a 0.20 μm filter (CA-S, Whatman) to prepare the CFCSs. The antifungal activity of the CFCSs was tested by microplate inhibition analysis with minor modifications (Ruggirello et al., 2019). Briefly, 140 μL of the CFCSs was added to 10 μL of the spore suspension (10^4 spores/mL) in each well. The positive control (PC) was 10 μL of the spore suspension (10^4 spores/mL) inoculated in 140 μL of fresh MRS broth in each well. Then, 150 μL of sterile fresh MRS broth was added in each well and was also used alone as the blank well (B). The microplates were then incubated at 28 °C for 72 h. All samples were analyzed in triplicate. The results were reported as a percentage calculated based on the OD_{490nm} values obtained from mold growth in the CFCS with the B values subtracted out as compared to the PC.

The stability of the antifungal activity of these strains was characterized based on the sensitivity of the CFCSs towards changes in pH, heat, and lytic enzymes, as previously described with some modifications (Sangmanee & Hongpattarakere, 2014). In order to test the pH sensitivity, the pH of the CFCSs was adjusted with 1 M NaOH or 1 M HCl to pH 4, 5, 6, and 7. The heat sensitivity was evaluated by subjecting the CFCSs to a number of treatments, including 63 °C for 30 min, 100 °C for 30 min, and 121 °C for 30 min. The sensitivity of the CFCSs to the activity of lytic enzymes was investigated by incubating the CFCSs with catalase, proteinase-K, and trypsin (Sigma-Aldrion Chemie, Shanghai, China) at a final concentration of 1 mg/mL for each for 3 h at 37 °C, respectively. The activity of the added enzymes was stopped by boiling the mixture for 5 min. All CFCS treatments were performed triplicate in a sterile 96-well microtiter plate.

2.5. Identification and quantification of antifungal compounds by HPLC and LC-MS

CFCSs of *L. kefiri* M4 and *P. acidilactici* MRS-7 were prepared, filtered through a 0.20 μm filter (CA-S, Whatman), and then poured into sample vials for analysis. All samples were analyzed in triplicate. Organic acids were determined via HPLC as previously described (Wei et al., 2020) with minor modifications. A Shimadzu LC-2030 PLUS system (Kyoto, Japan) equipped with a Shim-pake VP-DOS C_{18} analytical column (250 mm × 4.6 mm, particle size 4.6 μm; Shimadzu, Kyoto, Japan) was used. The mobile phase was 0.01 mol/L ammonium phosphate (Sigma Aldrich, Shanghai, China) (pH adjusted to 2.7 by phosphoric acid), and the flow rate was 0.7 mL/min. The separation was performed at 35 °C and detected using a wavelength at 210 nm.

CFCSs of *L. kefiri* M4 (1 mL) and *P. acidilactici* MRS-7 (1 mL) and acetone (1 mL) (Sigma Aldrich, Shanghai, China) were added into a test tube and mixed thoroughly. The mixture was incubated at 4 °C for 2–4 h, and then the mixture was centrifuged at 12,000×*g* for 10 min. The supernatant was collected and filtered with a 0.20 μm filter (CA-S, Whatman) for further analysis. LC-MS was performed on a Orbitrap Q-Extractive Focus System (Thermo Fisher, United States) equipped with a Hypersill GOLD C_{18} column (100 mm × 2.1 mm, 1.9 μm). The mobile phase for elution was performed using a binary solvent gradient (A: H_2O + 0.1% ammonium hydroxide (Sigma Aldrich, Shanghai, China), B: acetonitrile (Sigma Aldrich, Shanghai, China) + 0.1% ammonium hydroxide), as described in Table S1, with a flow rate of 0.2 mL/min. The total run time was 34 min, and the injection volume was 2 μL. The mass spectrometer was operated in negative electrospray ionization mode.

2.6. Production of lytic enzymes

LAB that produce lytic enzymes, such as chitinases, cellulases, proteases, and glucanases, were qualitatively detected using a solid agar amended with the various substrates. To assess cellulolytic and glucanolytic activity, *L. parakefiri* M1 and *L. kefiranofaciens* M3 were spotted on modified MRS agar plates containing 1% (w/v) cellulose and 0.5% (w/v) laminarin (Sigma Aldrich, Shanghai, China) (Wallace, Hirkala, & Nelson, 2017). After incubating under anaerobic conditions for 24 h, Congo red reagent (2 g/kg; Sigma Aldrich, Shanghai, China) was added to the modified MRS agar. After 30 min, the medium was decolorized with 1 M NaCl (Haibo, Qingdao, China) to observe the halo zones around the bacterial colonies (Lee et al., 2015). To assess chitinolytic and proteolytic activity, *L. parakefiri* M1 and *L. kefiranofaciens* M3 were spotted on modified MRS agar plates containing 1% (w/v) colloidal chitin (Sigma Aldrich, Shanghai, China) and 2% (w/v) skim milk agar. After incubating under anaerobic conditions for 24 h, the medium was evaluated in order to identify colonies that had a surrounding halo zone, the experiment was repeated 3 times.

2.7. Nutrient competition assay

Competition experiments were tested in 24-well culture plates with cylinder inserts with a hydrophilic polytetrafluoroethylene (PTFE) membrane (Nunc 140620, Thermo, USA) (Janisiewicz et al., 2000). Then, 300 μL of (1%, 5%, 10% v/v) apple juice (14 °Brix) with 300 μL of LAB (10^6 CFU/mL) or 300 μL of sterile water was added into the culture plates, and 400 μL of *P. expansum* (10^6 spores/mL) was added to the cylinder inserts. The cylinders were then placed in the wells and the plates were incubated at 28 °C. After 24 h, at least 100 spores per replicate were observed by light microscopy to evaluate the germination rate and germ tube length. The growth of LAB was evaluated by plating 10-fold serial dilutions on MRS plates. The plates were incubated at 37 °C for 2 days, and then the colonies were counted, the experiment was repeated 3 times.

2.8. Statistical analysis

For all the analyses, the data were analyzed statistically the SPSS statistical package version 22.0 (SPSS Inc., Chicago, IL) and Origin 9.0 (Origin Lab Corporation, USA). Analysis of variance (ANOVA, Duncan's method at a significance level of $p < 0.05$) was applied to the experimental data of antifungal activities of CFCSs and nutrient competition assay, Fisher's least significant difference test ($p < 0.05$) was used for HPLC analysis. Thermo Xcalibur Qual Browser 2.0 was used for the LC-MS raw data analysis.

3. Results and discussion

3.1. LAB isolation and identification

Kefir is rich in microbial resources. In this study, we isolated two LAB, *L. kefiri* M4 and *P. acidilactici* MRS-7, from kefir. Identification of the two strains was determined by a BLAST analysis of the partial sequences of the 16S rRNA gene. The results showed that *L. kefiri* M4 and *P. acidilactici* MRS-7 displayed 99% and 100% identity, respectively, to known sequences. The 16S rRNA gene nucleotide sequences for both LAB strains were deposited in NCBI database under the accession numbers MW082802 and MW082803. In addition, the taxonomic positions of *L. kefiri* M4 and *P. acidilactici* MRS-7 biotypes using 16S rRNA gene sequence homology are shown in Fig. 1. Similar to this work, Leite et al. (2012) found *L. kefiranofaciens* and *L. kefiri* were the dominant bacteria in three Brazilian kefir grains, while *Lactococcus*, *Leuconostoc*, *Streptococcus,* and *Acetobacter* were at low levels (Leite et al., 2012). This reveals that *Lactobacillus* species dominate kefir grains.

3.2. In vitro assays

Kefir has been demonstrated to inhibit plant pathogens. Ricardo Gamba, De Antoni, and Leon Pelaez (2016) reported that kefir products inhibited *Aspergillus flavus* growth (Ricardo Gamba, Andres Caro et al., 2016), and the CFCSs obtained from whey permeate fermented with kefir grains inhibited the growth of *Fusarium graminearum* and zearalenone production (Ricardo Gamba, De Antoni, & Leon Pelaez, 2016). While, there are few studies reporting the antifungal activity of LAB isolated from kefir. In our study, the qualitative analysis of the antifungal activity of *L. kefiri* M4 and *P. acidilactici* MRS-7 by the overlay method was shown in Table 1. Both strains showed the strongest inhibition against *P. expansum* LPH9 and *P. expansum* LPH10 and exhibited no inhibition against *P. expansum* LPH6. *L. kefiri* M4 had weak inhibition against *P. expansum* F-WY-12-02, while *P. acidilactici* MRS-7 exhibited strong inhibition against *P. expansum* F-WY-12-02 and weak inhibition against *Penicillium expansum* CGMCC3.3703. Our results showed that the resistance of pathogens was different, although they were in the same species. Similarly, Rodríguez-Chávez, Juárez-Campusano, Delgado, and Pacheco Aguilar (2019) reported that LAB strains isolated from kefir showed a fairly good antifungal effect against *P. expansum* (Rodríguez-Chávez et al., 2019). *L. kefiri* was also shown to significantly inactivate *Listeria monocytogenes* when galactooligosaccharide (GOS) was added to the co-culture medium (Likotrafiti, Valavani, Argiriou, & Rhoades, 2015). Moreover, multiple reports have identified antimicrobial LAB strains from various environmental samples, including herbs, fresh fruits, vegetables, flowers, soil, and leaves (Cheong et al., 2014).

3.3. Characterization of the antifungal activities of CFCSs

LAB can produce a variety of bioactive compounds such as cyclic peptides, reuterin, organic acids, hydrogen peroxide, phenolic antioxidants, diacetyl and so on (Sadiq et al., 2019). The chemical properties of these compounds determine their uses, for example, many antifungal peptides have obtained much interest by researchers due to their properties such as high stability at various pH and temperatures thermal

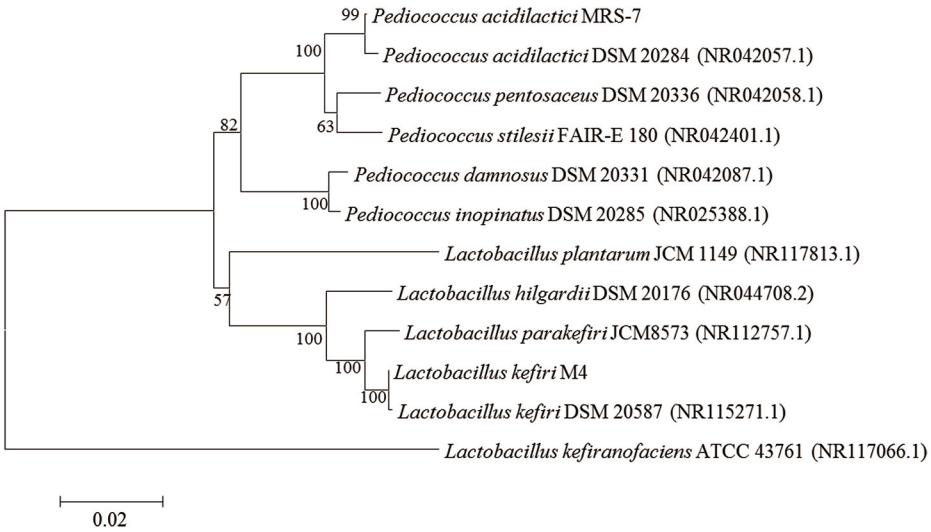

Fig. 1. Phylogenetic tree analysis of *L. kefiri* M4 and *P. acidilactici* MRS-7 using the neighbor-joining algorithm. The numbers above the branches are confidence limits expressed as percentages (estimated from the bootstrap analyses performed with 1000 replicates). The scale bar represents 0.02% sequence divergence.

Table 1
Antifungal activity spectrum of *L. kefiri* M4 and *P. acidilactici* MRS-7.

Mold strains	Antifungal activity	
	L. kefiri M4	*P. acidilactici* MRS-7
P. expansum F-WY-12-02	+	+++
P. expansum LPH6	–	–
P. expansum LPH9	+++	+++
P. expansum LPH10	+++	+++
P. expansum CGMCC3.3703	+++	+

The results are expressed as "strong inhibition (+++), medium inhibition (++), weak inhibition (+), no inhibition (−)".

stability (Thery, Lynch, & Arendt, 2019). Some researchers have reported the use of LAB antifungal peptides in the prevention of wheat grains and maize seeds spoilage under storage (Juodeikiene et al., 2018; Muhialdin et al., 2020). In order to characterize the nature of active molecules in CFCSs of both stains, various treatments with heat, proteases, and pH neutralization were performed.

The antifungal activity of the isolated LAB CFCS against *P. expansum* LPH10 was 100% (data not shown). The activity of *L. kefiri* M4 and *P. acidilactici* MRS-7 decreased when the CFCSs were adjusted from pH 4.0 to 6.0. When the supernatant of both strains was adjusted to pH 5, the inhibitory activity of *L. kefiri* M4 and *P. acidilactici* MRS-7 against *P. expansum* LPH10 decreased to 47.4% and 52.6% (Fig. 2A), respectively. Both strains had no effect on *P. expansum* LPH10 at pH 6.0. Similar to this, when *L. plantarum* K35 supernatant was neutralized from pH 4.0 to 7.0, the inhibitory activity against *A. flavus* and *A. parasiticus* decreased, with MIC values of 11 mg/mL and 22 mg/mL to 88 mg/mL and 176 mg/mL, respectively (Sangmanee & Hongpattarakere, 2014). Guimaraes, Santiago, Teixeira, Venancio, and Abrunhosa (2018) reported that the inhibitory effect of CFCSs on fungal growth dropped to 0% upon neutralization (Guimaraes et al., 2018). Additionally, when the CFCSs were neutralized to pH 7.0, the growth of *P. expansum* LPH10 decreased for their growth conditions approaches the end of the domain. As for heat treatments, the antifungal activity of the CFCSs remained stable after heating at 63 °C for 30 min and 100 °C for 30 min but decreased after heating at 121 °C for 30 min (Fig. 2B). However, the antifungal substances of *L. plantarum* K35 remained active at 121 °C for 15 min (Sangmanee & Hongpattarakere, 2014). Le Lay et al. (2016) reported that the antifungal activities of CFCSs were affected by heating at 90 °C for 20 min, except for the activity of *L. citreum* L123 against *P. corylophilum* UBOCC-A-112081, which was conserved, but was lost after heating at 120 °C for 20 min (Le Lay et al., 2016). Finally, none of the CFSCs were affected by enzymatic action (data not shown), suggesting that no proteinaceous compounds contributed to the antifungal activity of *L. kefiri* M4 and *P. acidilactici* MRS-7. Taken together, our results showed that organic acids were the main element responsible for the antifungal effect, and thermosensitive molecules partly contributed the antifungal activities of *L. kefiri* M4 and *P. acidilactici* MRS-7.

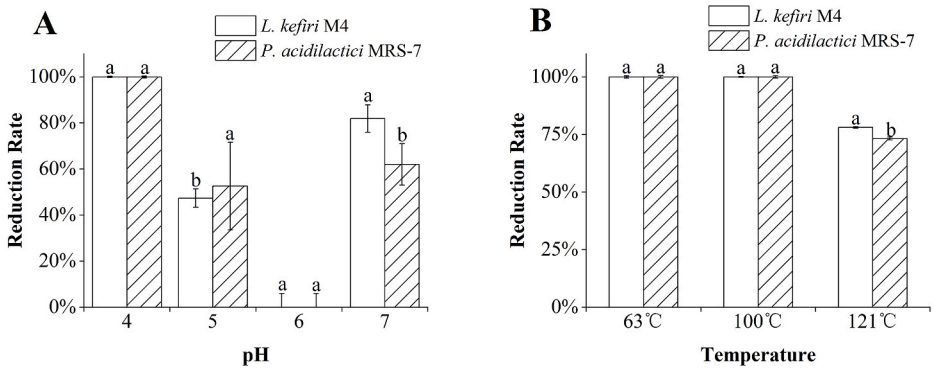

Fig. 2. Effect of pH treatment (A) and heat treatment (B) on the antifungal activity of *L. kefiri* M4 and *P. acidilactici* MRS-7 against *P. expansum* LPH10. Columns with different lowercase letters within the same panel are significantly different ($P < 0.05$) according to Duncan's multiple range test.

3.4. Detection of the chemical composition of CFCSs

In this study, we quantitatively analyzed eight organic acids in the supernatant by HPLC analyses. As shown in Table 2, among those acids, both strains produced a high concentration of lactic acid (9.776 g/L and 10.146 g/L, respectively). Both malic acid and fumaric acid were not detected. The concentration of formic acid, oxalic acid, tartaric acid and citric acid produced by both strains was comparable. *P. acidilactici* MRS-7 produced the highest concentration of propanoic acid (4.396 g/L), which was almost 10 times more than that in the *L. kefiri* M4 supernatant. Additionally, acetic acid (12.343 g/L) and succinic acid (1.363 g/L) produced by *P. acidilactici* MRS-7 were almost twice as high as that measured in the supernatant of *L. kefiri* M4.

LAB produce weak organic acids such as lactic, acetic, and propionic acid as fermentation end products, creating an acidic environment that generally restricts growth of spoilage fungi (Ross, Morgan, & Hill, 2002). Corsetti, Gobbetti, Rossi, and Damiani (1998) found a mixture of organic acids, including acetic acid, caproic acid, formic acid, propionic acid, butyric acid, and n-valeric acid, were synergistically responsible for the fungal inhibitory effect of *L. sanfranciscensis* CB1, which caused a strong inhibition of *A. niger* 10547, *F. graminearum* 623, *P. expansum* FS2 and *M. sitophila* FS5, but no inhibition to *F. graminearum* 623 when the acids were tested individually at the concentrations produced by *L. sanfranciscensis* (Corsetti et al., 1998). This suggests organic acids might inhibit fungal growth through synergistic or additive effects. Besides, some carboxylic acids were identified as antifungal compounds from LAB, such as p-couramic, azealic, benzoic, 4-hydroxybenzoic acid, vanillic, hydrocinnamic, and hydroxybenzoic acids (Broberg, Jacobsson, Strom, & Schnurer, 2007; Brosnan, Coffey, Arendt, & Furey, 2012).

To identify more antifungal compounds produced by LAB, higher sensitivity and more efficient separation was the key to the detection of low concentrations of organic compounds in complex solutions. In this study, LC-MS was applied to detect and identify antifungal compounds. We searched for 23 antifungal compounds reported in the literature, and all of them were detected in the supernatant of both strains, except for 1,2-dihydroxybenzene and hydrocinnamic acid D9 (Table 3, Fig. S2). Similarly, Le Lay et al. (2016) reported that azelaic acid, phenyllactic acid, (S)-(−)-2-hydroxyisocaproic acid, DL-ρ-hydroxyphenyllactic acid, vanillic acid, and cytidine were detected in the supernatant of *Leuconostoc citreum* L123 (Le Lay et al., 2016). Honore et al. (2016) found 2-hydroxy-4-methylpentanoic acid, 2-hydoxy-3-phenylpropanoic acid, and 2-hydoxy-3-(4-hydroxyphenyl)propanoic acid were the major metabolites of *L. paracasei* by LC-MS (Honore et al., 2016).

Table 2
Quantification of organic acids produced in the supernatants of *L. kefiri* M4 and *P. acidilactici* MRS-7 by HPLC.

Compounds (g/L)	Strain	
	L. kefiri M4	*P. acidilactici* MRS-7
Lactic acid	9.776 ± 0.016[b]	10.146 ± 0.112[a]
Formic acid	3.914 ± 0.228	3.982 ± 0.098
Acetic acid	5.578 ± 0.175[b]	12.343 ± 0.291[a]
Propanoic acid	0.424 ± 0.049[b]	4.396 ± 0.427[a]
Oxalic acid	0.516 ± 0.006	0.535 ± 0.017
Tartaric acid	2.508 ± 0.027	2.542 ± 0.075
Malic acid	ND	ND
Citric acid	1.657 ± 0.095	1.772 ± 0.046
Fumaric acid	ND	ND
Succinic acid	0.643 ± 0.196[b]	1.363 ± 0.047[a]

Results are expressed as the mean of three replicates ± standard deviation. Within the same row, means with different letters are significantly different according to a Fisher's least significant difference test ($P < 0.05$). ND: compounds not detected.

3.5. Production of lytic enzymes

In addition to antifungal compounds, the activity of lytic enzymes may be attributable to the antifungal properties of LAB. In this study, both strains were positive for proteases, cellulases, and glucanses, but negative for chitinase (Fig. S3), supporting that both strains have the ability to degrade of fungal hyphae. However, there are few reports about the lytic enzymes of LAB, and we only found that J.-S. Lee isolated three strains *Lactobacillus pentosus* PL11, PL13, and PL16 (Anguilla japonica), which produces celluloses and proteases (Lee et al., 2015).

3.6. Competition for nutrients

The competition of nutrients *in vitro* was researched by tissue culture plate insert, in which the cylinder inserts contained a PTFE membrane that allowed liquid to move rapidly through it and was suitable for the microscopic observation of the conidia and germ (Janisiewicz et al., 2000). The culture plate offers the same nutrients and enough space for the antagonist and pathogen to interact indirectly. In this study, the nutrient competition results between *L. kefiri* M4 and *P. expansum* LPH10 are presented in Fig. 3. The population of *L. kefiri* M4 (Fig. 3A) and the spore germination rate of *P. expansum* LPH10 increased with the increase in apple juice concentration. *L. kefiri* M4 supernatant prevented the germination of the spores in apple juice, with the highest inhibitory activity in 2.5% apple juice (Fig. 3B), then the inhibition activity decreased as the concentration continue increased compared with 2.5% apple juice. The result is consistent with the previous studies (Bencheqroun et al., 2007; Janisiewicz et al., 2000; Meziane et al., 2006). This revealed that when the nutrient concentration was low, antagonistic bacteria preferentially utilized the nutrition, resulting in no enough nutrition for the pathogen germination. When the nutrient concentration was increased, it can not only meet the growth and reproduction needs of antagonistic bacteria, but also meet the germination needs of pathogenic spore, so the pathogenic spore can germinate normally. When the cylinders containing ungerminated conidia were transferred to wells without *L. kefiri* M4 for another 2 days, the spore germination rate increased as the concentration of the apple juice increased, with a maximum spore germination rate of 88.67% in 5% apple juice. This revealed that the pathogenic spore did not die from not having enough nutrition, but that germination was temporarily inhibited. However, after transferring to a high concentration of apple juice, the germination rate failed to recover to the untreated level, which is consistent with a previous study (Bencheqroun et al., 2007). Therefore, it was revealed that the metabolites produced by antagonistic bacterium caused damage to pathogenic spores.

4. Conclusion

In conclusion, the current study shows that *L. kefiri* M4 and *P. acidilactici* MRS-7 isolated from kefir have antifungal activity against most *Penicillium expansum*. The characterization of the antifungal activities of CFCSs shows that both strains supernatant are pH-dependent, partly heat sensitive, and are not influenced by proteinaceous treatment, which may help in understanding the antifungal activity of these LAB. HPLC and LC-MS suggest the main antifungal compounds of CFCSs were considered as organic acids and carboxylic acids. However, more unknown antifungal compounds need to be further identified. Additionally, we observed nutrient competition when *L. kefiri* M4 and *P. expansum* LPH10 were co-cultured. In short, the possible antifungal mode of action of both strains may be related to the production of antifungal compounds, lytic enzymes and nutrient competition, deeper antifungal mechanism should be explored in the future.

CRediT authorship contribution statement

Hong Chen: Conceptualization, Data curation, Formal analysis,

Table 3
Name, chemical formula, theoretical mass [M-H]⁻, detected mass [M-H]⁻, retention time and calculated ppm error for antifungal compounds detected in the supernatants of *L. kefiri* M4 and *P. acidilactici* MRS-7.

Antifungal compound	Chemical formula	[M-H]⁻theoretical m/z	*L. kefiri* M4			*P. acidilactici* MRS-7		
			[M–H]⁻found m/z	Retention time (min)	ppm error	[M–H]⁻found m/z	Retention time (min)	ppm error
Cytidine	$C_9H_{13}N_3O_5$	242.07824	242.07822	1.38	0.08	242.07819	0.86	0.21
2-Deoxycytidine	$C_9H_{13}N_3O_4$	226.08333	226.08303	1.13	1.33	226.08310	1.38	1.24
D-Glucuronic acid	$C_6H_{10}O_7$	193.03538	193.03552	24.09	−0.73	193.03580	12.39	−2.26
1,2-Dihydroxybenzene	$C_6H_6O_2$	109.02950				109.03050	24.16	−8.71
3,4-Dihydroxyhydrocinnamic acid	$C_9H_{10}O_4$	181.05063	181.05020	32.66	2.38	181.04980	20.52	4.36
4-Hydroxybenzoic acid	$C_7H_6O_3$	137.02442	137.02509	15.59	−4.89	137.02520	13.06	−5.55
Caffeic acid	$C_9H_8O_4$	179.03498	179.03506	27.49	−0.45	179.03430	14.19	3.80
Vanillic acid	$C_8H_8O_4$	167.03498	167.03510	13.54	−0.72	167.03410	1.57	5.51
(S)-(−)-2-Hydroxyisocaproic acid	$C_6H_{12}O_3$	131.07137	131.07185	2.33	−3.66	131.07010	32.24	9.38
3-(4-Hydroxyphenyl)propionic acid	$C_9H_{10}O_3$	165.05572	165.05516	31.19	3.39	165.05480	20.08	5.45
3-(4-Hydroxy-3-methoxyphenyl) propanoic acid	$C_{10}H_{12}O_4$	195.06628	195.06627	23.76	0.05	195.06530	15.20	4.82
p-Coumaric acid	$C_9H_8O_3$	163.04007	163.03906	1.28	6.19	163.03920	0.76	5.64
Ferulic acid	$C_{10}H_{10}O_4$	193.05063	193.04967	16.16	4.97	193.04910	32.54	8.08
Azelaic acid	$C_9H_{16}O_4$	187.09758	187.09717	33.66	2.19	187.09700	25.92	3.10
Phenyllactic acid	$C_9H_{10}O_3$	165.05572	165.05516	31.19	3.39	165.05480	20.08	5.45
Benzoic acid	$C_7H_6O_2$	121.02950	121.02988	29.82	−3.14	121.02980	26.67	−2.64
Hydrocinnamic acid	$C_9H_{10}O_2$	149.06080	149.05962	4.00	7.92	149.05960	24.01	7.85
Methylcinnamic acid	$C_{10}H_{10}O_2$	161.06080	161.05956	32.78	7.70	161.05960	32.45	7.64
3-Hydroxydecanoic acid	$C_{10}H_{20}O_3$	187.13397	187.13329	20.10	3.63	187.13340	33.04	3.31
Decanoic acid	$C_{10}H_{20}O_2$	171.13905	171.13824	0.78	4.73	171.13830	26.49	4.32
DL-β-Hydroxymyristic acid	$C_{14}H_{28}O_3$	243.19657	243.19658	20.01	−0.04	243.19660	15.13	0.04
2-Hydroxydodecanoic acid	$C_{12}H_{24}O_3$	215.16527	215.16427	14.67	4.65	215.16500	4.21	1.16
Hydrocinnamic acid D9	$C_9HO_2D_9$	158.11729						

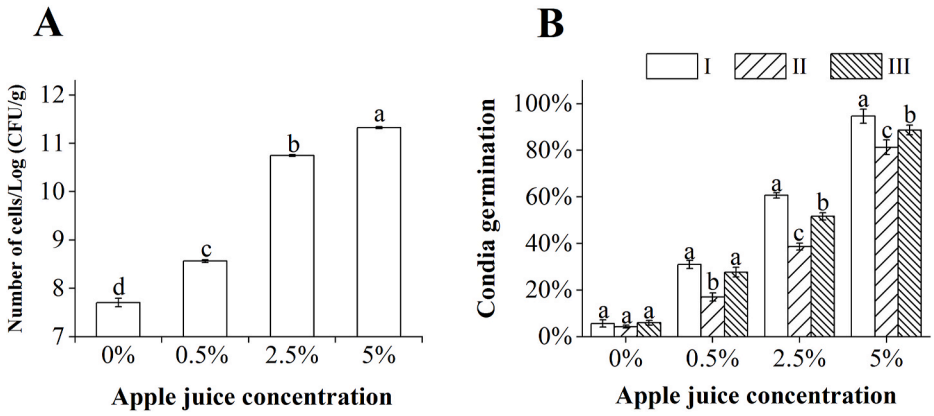

Fig. 3. Growth of *L. kefiri* M4 in different concentrations of apple juice (A) and its effect on conidia germination (B). (I: without *L. kefiri* M4 for 24 h; II: with *L. kefiri* M4 for 24 h; III: with *L. kefiri* M4 for 24 h first and then without *L. kefiri* M4 for another 24 h). Columns with different lowercase letters within the same panel are significantly different ($P < 0.05$) according to Duncan's multiple range test.

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Declaration of competing interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2021.108274.

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EFFECT OF LACTIC ACID BACTERIA AS BIO-PRESERVATION AGAINST SPOILAGE FUNGI FROM PAPAYA FRUIT

Antifungal preservation of food by lactic acid bacteria / Nasrollahzadeh, A., Mokhtari, S., Khomeiri, M., & Saris, P. E. J.

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Review

Antifungal Preservation of Food by Lactic Acid Bacteria

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Abstract: Fungal growth and consequent mycotoxin release in food and feed threatens human health, which might even, in acute cases, lead to death. Control and prevention of foodborne poisoning is a major task of public health that will be faced in the 21st century. Nowadays, consumers increasingly demand healthier and more natural food with minimal use of chemical preservatives, whose negative effects on human health are well known. Biopreservation is among the safest and most reliable methods for inhibiting fungi in food. Lactic acid bacteria (LAB) are of great interest as biological additives in food owing to their Generally Recognized as Safe (GRAS) classification and probiotic properties. LAB produce bioactive compounds such as reuterin, cyclic peptides, fatty acids, etc., with antifungal properties. This review highlights the great potential of LAB as biopreservatives by summarizing various reported antifungal activities/metabolites of LAB against fungal growth into foods. In the end, it provides profound insight into the possibilities and different factors to be considered in the application of LAB in different foods as well as enhancing their efficiency in biodetoxification and biopreservative activities.

Keywords: synthetic preservatives; preservation enhancement; metabolites; supplementation with LAB

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1. Introduction

Fungi are among the most serious food-spoiling micro-organisms threatening the quality and health of food, food products, and feed [1]. Fungal plant-pathogens destroy up to 30% of crop products, and spoiling fungi and their toxins contaminate about 25% of raw materials produced by agriculture worldwide [2]. It is estimated that the annual economic loss caused by the spoilage of bread by fungi will reach to more than EUR 200 million in Western Europe [3].

The disadvantages of using synthetic preservatives such as the formation of carcinogenic nitrosamines in food are well known, though mold species are also becoming resistant to them [4,5]. The biopreservation of food products by natural and biological compounds may be a satisfactory alternative to solving microbial spoilage of food and food products and its consequent economic loss, which will also contribute to reducing the incidence of foodborne illnesses [6].

According to extensive studies in recent decades, LAB being able to produce active compounds such as fatty acids, organic acids, hydrogen peroxide, peptides, and reuterin represent ideal biopreservatives for conventional chemical antifungal preservatives against spoilage and toxigenic compounds in food [7,8]. A total of 25% of Europe's diet and 60% of the diet of many developing countries is composed of fermented food, and LAB play a great role in the fermentation process [9,10]. In addition, LAB cultures isolated from native fermented food products with probiotic attributes and mycotoxin binding may be of immense value in decontaminating mycotoxins in food [11,12].

This review aimed to summarize the capability of LAB as green preservatives in different foods by highlighting their antifungal substances and mechanisms of their action. Moreover, foodborne diseases caused by pathogenic fungi as well as the hazards of synthetic preservatives for human health were outlined. Finally, a comprehensive insight into various aspects of the application of LAB as biopreservatives in foods was provided.

2. Foodborne Diseases

Foodborne diseases (also called foodborne infection or food poisoning) comprise a wide spectrum of diseases resulted from the ingestion of foodstuff spoilage or pathogen microorganisms and toxic chemicals. Foodborne diseases count as a considerable cause of morbidity and mortality, which subsequently pose a remarkable impediment to socioeconomic development all around the world [13]. Since many different pathogenic microorganisms can contaminate food, there is a wide variety of foodborne infections. The Centers for Disease Control and Prevention (CDC) estimated that each year in the United States of America, 48 million people become sick as a result of foodborne illness, 128,000 people are hospitalized, and 3000 people die [14]. According to the WHO, unsafe food causes 600 million cases of foodborne diseases and 420,000 deaths annually worldwide, of which 30% belong to children under 5 years of age. The WHO estimated that eating unsafe food leads to the loss of 33 million years of lives globally each year [15]. The production and release of mycotoxins in food is the most important and dangerous effect caused by fungi to human health [16].

3. Synthetic Preservatives and Hazards of Their Use

Synthetic preservatives are substances of chemical origin that inhibit the growth of spoilage microorganisms. Some examples are benzoates, sorbates, propionate, EDTA, nitrites, and sulfites [17]. The majority of preservatives used today are synthetic rather than natural, and several of them potentially pose life-threatening side effects over time for humans as well as negative impacts on the environment [18]. Researchers have reported that synthetic preservatives can cause serious health hazards such as cancer, allergy, asthma, hyperactivity, and damage to the nervous system [19,20]. A scientific report described the cumulative behavioral effects of bread preservative on children. Daily consumption of preservative in foods has the potential to cause irritability, restlessness, inattention, and sleep disturbance in children [21]. Table 1 shows the most common synthetic antifungal preservatives, their negative effect on human health, and fungi that have developed partial resistance to them.

Table 1. The most common synthetic antifungal preservatives, their negative effect on human health, and fungi that have developed partial resistance to them.

Preservatives	Food	Health Effects	Resistant Fungi	References
Benzoate	Fruit products Acidic foods Margarine Cereals Meat Carbonated drinks	Neurotransmission and cognitive functioning Hyperactivity and allergic reactions Genotoxic Clastogenic intercalation in the DNA structure		[19] [22] [23]
			Zygosaccharomyces bailii	[24]
			Aspergillus flavus	[25]
			Aspergillus niger and *Penicillium notatum*	[5]
			Elymus repens and *A. niger*	[26]
			Aspergillus conicus, *Penicillium*, *Cladosporium* and *Wallemia*	[27]
Propionate	Breads and other baked goods	Hypersensitivity Visual irritability Restlessness Inattention Sleep disturbance		[19]
			E. repens and *A. niger*	[26]
			A. conicus, *Penicillium*, *Cladosporium* and *Wallemia*	[27]
			Penicillium expansum and *Penicillium roqueforti*	[28]
			P. roqueforti	[29]

Table 1. *Cont.*

Preservatives	Food	Health Effects	Resistant Fungi	References
Sorbate	Syrups Dairy products Cakes Mayonnaise Margarine Processed meats	Cytotoxic and genotoxic effects DNA breakage Irritant to respiratory epithelium	*P. roquefortii*	[19] [30] [31] [32]
			A. flavus	[32]
			P. notatum and *A. niger*	[5]
			Rhizopus nigricans	[4]
			E. repens and *A. niger*	[26]
			A. conicus, *Penicillium*, *Cladosporium* and *Wallemia*	[27]

Benzoates mainly inhibit mold, yeasts, and bacteria in liquid environments such as acidic and soft drinks. Sodium benzoate is the most common salt of benzoate used in carbonated drinks, fruit juices, and some other foods with a pH of 3.6 or lower. It is established that benzoate can react with ascorbic acid in drinks and produce benzene, which is a carcinogen [17]. It is also reported to influence neurotransmission and cognitive functioning [33]. Although sodium benzoate is regarded as safe by major regulatory agencies, there are still questions over its adverse effects on human health. Sodium benzoate intake of above 5 mg/kg resulted in allergy and hyperactivity. Sodium benzoate has the potential to cause changes in the cell cycle and impairment in DNA as well as being considered as genotoxic and clastogenic [34].

Propionates inhibit mold growth in baked goods [17]. Although regarded as GRAS by FDA, there is still a lack of clarity on the metabolic effects of propionate in humans. Propionate may cause hyperinsulinemia, promoting adiposity and metabolic abnormalities over time [35]. Propionate preservatives are also reported to contribute to or cause visual irritability, restlessness, inattention, and sleep disturbance in some children [21].

Sorbates prevent mold/yeast growth in food products [17]. Even though sorbate is legally used in the food industry, it still has the potential to cause harmful side effects if consumed in quantities higher than the standard limits or if used long-term [36]. Various research results showed that the increased potassium sorbate intake above 25 mg/kg may lead to producing mutagenic compounds and inducing chromosome damage and DNA breakage and irritation to the respiratory epithelium [36,37].

Apart from negative impacts on health, synthetic preservatives may also adversely affect the organoleptic properties of the food. One of the serious problems in cheese preserved with sorbate is the decomposition of sorbic acid and potassium sorbate to trans-1, 3-pentadiene by resistant strains, and the consequent undesirable taste and odor in cheese, known as kerosene [38,39]. According to Ferrand et al. [40], sorbates might influence the taste of the food, though they are physiologically harmless and less toxic compared to benzoates [40].

Some fungi and yeasts have acquired the ability to resist chemical treatments and preservatives, which consequently creates the demand for a higher dose of the preservatives to be used. Frequent use of common antifungal agents is blamed for causing mutation in the target microorganisms and increasing their resistance [41,42]. It has been reported that some *Penicillium*, *Saccharomyces*, *Zygosaccharomyces*, *Rhizopus*, and *Yarrowia* strains can grow in the presence of potassium sorbate [4,5,43,44]. Additionally, *Z. bailii* and *P. roqueforti* isolates have been reported to be resistant to and even degrade benzoate, respectively [23,44]. These facts together with the demand for least processed foods and the potential hazards of synthetic preservative usage have directed the research sector for seeking alternatives for food preservation.

4. Lactic Acid Bacteria (LAB)

LAB homofermentatives are the species that produce lactic acid as the sole final product, while the heterofermentative ones produce lactic acid, CO_2, and ethanol or acetate. At least half of the final product carbon is a form of lactate [45].

For centuries, LAB have been employed as bacteria performing a central role in a diversity of fermented foods involving milk, vegetables, meats, and sourdough by inducing rapid acidification of the raw material [46,47]. When it is used regularly, LAB-fermented food confers health benefits by strengthening the body in the battle with pathogenic bacterial infections [48].

LAB have also received considerable attention as probiotics over the past few years. Improving health by the biotransformation of different compounds in the gastrointestinal tract into bioavailable ones such as vitamins and short-chain fatty acids by LAB have been reported [49,50]. Immune modulation, anticarcinogenic and antitumor activity, the reduction of cholesterol, alleviation of lactose intolerance, normalization of stool transit, hepatic encephalopathy, and treatment of peptic ulcers are a number of health benefits and indicate the safety of probiotics LAB. Additionally, some modes of action of probiotic LAB are acid tolerance, adhesion to mucus and epithelial cells, production of antimicrobial compounds, and immune stimulation [51–53].

4.1. LAB as Green Preservatives in Food Systems

Fermentation of some foods by LAB strains with antifungal properties has been demonstrated to reduce chemical preservative usage in the food. According to Axel et al. [54], the use of sourdough fermented with specific strains of antifungal LAB can reduce chemical preservatives in bakery products [54].

LAB can be used as natural compounds to replace the chemical preservatives and are associated with health-promoting and probiotic properties [55]. LAB strains with antifungal activity also have the potential to work in synergy with synthetic preservatives. A combination of propionate and sorbate with acetic acid was shown to represent synergistic effects against fungal species of *P. roqueforti* and *A. niger* [56]. In another study, sourdough fermented by antifungal *Lactiplantibacillus plantarum* strains was studied for inhibition activity against *Fusarium culmorum*, *A. niger*, or *P. expansum* spores. Strong synergistic activity was reported when a combination of calcium propionate and the sourdoughs fermented by *L. plantarum* into the bread formulation was applied. The reduced use of calcium propionate up to 1000 ppm maintained inhibition only when the antifungal sourdough was added. Additionally, the increase in shelf life was interestingly higher than that obtained using calcium propionate alone (3000 ppm) [28].

In some research, in situ addition of LAB into food and feed was proven to delay fungal growth. Some examples are in fruits and vegetables, sour cream and semi-hard cheese, quinoa, and rice bread [54,57,58].

In situ application of LAB strains with antifungal activity in some foods have proven potential to act better than synthetic preservatives and competency to replace them in the foods. Rice dough fermented by some LAB isolated from kimchi resisted against three fungal species of *Cladosporium* sp. YS1, *Penicillium crustosum* YS2, and *Neurospora* sp. YS3 much better than that of 0.3% calcium propionate [59]. One *Leuconostoc* and five *Lactobacillus* strains surface sprayed on bakery products were shown to delay the growth of some resistant and semi-resistant fungi to calcium propionate, potassium sorbate, and sodium benzoate [26]. In the study of Mandal, Sen, and Mandal [60], the antifungal compound of *Pediococcus acidilactici* LAB 5 at a high dilution (0.43 mg mL^{-1}) exerted a greater inhibition of *Curvularia lunata* conidia than sodium benzoate [60]. Valerio et al. [61] also reported that *Leuconostoc citreum*, *Weissella cibaria*, and *Lactobacillus rossiae* isolated from Italian durum wheat semolina inhibit fungal strains of *A. niger*, *P. roqueforti*, and *Endomyces fibuliger* to the same or a higher extent in comparison with calcium propionate. The results of the study indicated a potent inhibitory activity of the ten LAB strains used in their

study compared to that obtained with calcium propionate (0.3% *w*/*v*) against the most widespread contaminant of bakery products, *P. roqueforti* [61].

4.2. Antifungal Activity Spectrum of LAB

LAB have a reported potential use as adjunct or starter cultures to inhibit fungi growth in the final products such as fruit and vegetables, dairy, and bakery. Twenty LAB isolates from fermented cassava were investigated against fungal pathogens associated with the spoilage of vegetables and fresh fruits. Strong inhibition of the radial growth and spores of the fungal pathogens was observed when the products were inoculated with the antifungal metabolites produced by the strains [57].

In cheese, *Lactobacillus amylovorus* DSM 19280 was used as an adjunct culture in a cheddar cheese model system contaminated with *P. expansum* spores. The presence of the strain resulted in a four-day delay in *Penicillium* growth on the cheddar cheese compared with the control [62]. *Lactobacillus rhamnosus* A238 was also shown alone or in combination with *Bifidobacterium animalis* to inhibit mold growth on cottage cheese for at least 21 days at 6 °C [63]. In another study, 12 selected *L. plantarum* isolates were inoculated into cottage cheese challenged with *Penicillium commune*. All the isolates were found to prevent the obvious *P. commune* growth on cottage cheese by between 14 and more than 25 days longer than the control [64]. *Lactobacillus brevis* and *Enterococcus faecium* isolated from "chal", a product from yogurt, reduced the growth of *Rhodotorula glutinis* in doogh, diluted yogurt, over 15 days of storage [65].

In sour cream and semi-hard cheeses, Lactobacillus paracasei CIRM-BIA1759 and L. rhamnosus CIRM-BIA1761 were tested as adjunct cultures. In situ assays showed that the strains postponed the growth of *P. commune*, *Rhodotorula mucilaginosa*, and *Mucor racemosus* on sour cream for 2–24 days and also delayed the growth of *P. commune* in semi-hard cheese for 1–6 days [58]. Ouiddir et al. [66] tested the antifungal activity of *L. plantarum* CH1, *L. paracasei* B20, and *Leuconostoc mesenteroides* L1 in sour cream and sourdough bread challenged with fungal spoilers. The strains delayed the growth of the *Aspergillus tubingensis*, *A. flavus*, *P. commune*, and *M. racemosus* for up to 5 days in sourdough bread. In sour cream, *L. plantarum* CH1 and *L. paracasei* B20 completely inhibited *P. commune* growth for 5 and 3 days, respectively [66].

In bakery products, in situ sprays of one *Leuconostoc* and five *Lactobacillus* strains delayed one or several fungal species growths. The incorporation of the same strains in milk-bread-roll preparation also delayed fungal growths [26]. In another study, two strains of *Lactobacillus* were used for sourdough fermentation of quinoa and rice flour. *L. reuteri* R29 and *L. brevis* R2Δ fermented sourdough bread reached a shelf life of quinoa and rice from 2 to 4 days, respectively [54]. A Chinese steamed bread manufactured with *L. plantarum* CCFM259 did not show any fungal contamination until 7 days of storage, a similar level of inhibition compared with that obtained by 0.25% (*w*/*w*) calcium propionate [67]. Fermenting rice dough with some LAB isolated from kimchi greatly retarded the growth of three fungal species from *Cladosporium*, *Neurospora*, and *Penicillium* genus in the rice cakes [59].

Different LAB isolates have the potential to synergically inhibit fungal growth in food. Seven strains of LAB were selected and tested for their anti-penicillium activity to prevent *Penicillium chrysogenum* growth in cottage cheese. They found that some of the strains act in synergy, and their combination has potential for use as bio-preservatives in fresh cheese [63].

Antifungal activity of LAB depends on the pH, temperature, growth media, incubation time, nutrients, antifungal compounds, production levels, and mode of action [68]. Mandal, Sen, and Mandal [60] observed that the production of antifungal compound(s) from *P. acidilactici* LAB 5 against pathogenic fungi showed a great dependency on media specifications. TGE, and TGE + Tween 80 media did not support the production of any antifungal compounds, while the fungal growth was completely restricted in MRS agar media [60]. Another study reported that supplementation of WFH media with 2.5% olive

oil and 150 mM glycerol raised the antifungal activity of *L. brevis* Lu35 and *L. reuteri* 5529, respectively [26]. The addition of linoleic acid supported the antifungal activity of *Lactobacillus hammesii* [69]. Rouse et al. [70] reported that when grown in different carbon sources, the antifungal activity of the LAB strains tested was stable, although the quantity of metabolites produced varied depending on the carbon source. Among the sugars tested, for three out of four strains, glucose and lactose were the best and worst, respectively [70].

The incubation time has been observed to greatly influence the antifungal activity of LAB. Rouse et al. [70] observed that the four tested LAB cultures were unable to grow at 10 and 42 °C, and consequently, no growth was observed. Incubation between 21 and 37 °C, however, improved growth, and the bacteria presented different levels of antifungal activity with the optimal production of the antifungal compounds between 25 and 30 °C [70]. The antifungal activity of *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 was slightly better at 30 °C as compared to 25 °C for 40 h. The production of antifungal compounds by the strain was reported to begin in the log phase and reach a maximum level in the early stages of the stationary phase followed by a drop in activity [71].

The antifungal activity of LAB has also been found to be influenced by pH. The antifungal activity of *L. plantarum* K35 was reported to be pH-dependent and favorable to acidic conditions [72]. Antifungal properties of *L. coryniformis* subsp. *coryniformis* strain Si3 were also observed at maximum at pH values of between 3.0 and 4.5, with a decrease in pH between 4.5 and 6.0, and loss at higher pH values. Readjustment of the pH to 3.6 fully returned the activity [71]. In another study, antifungal attributes of the four LAB strains were found to be good at pH 3, moderate at pH 5, and low at pH 7, although poor fungal inhibition was maintained at pH 8 [73]. The effect of temperature and pH on the antifungal properties of the *L. plantarum* strain against *Aspergillus fumigatus* and *Rhizopus stolonifer* in temperatures ranging from 20 °C to 40 °C and pH ranging from 4.0 to 7.0 for 48 h of incubation was investigated. A combination of 30 °C and pH 6.5 °C presented optimum antifungal activity [74].

The role of the concentration of supernatant in the antifungal activity of LAB were also highlighted by Shehata et al. (2019), where they observed that increasing the supernatant concentration of *Lactobacillus* sp. RM1 decreased the growth of *Aspergillus parasiticus*, *A. flavus*, and *Aspergillus carbonarius* [18].

Fermentation time was also found to be effective in the antifungal activity of LAB. Longer fermentation times of barley malt substrate fermented by LAB resulted in higher carboxylic acids released by them against *F. culmorum* macroconidia. The maximal concentrations of the acids were obtained after 48 h of fermentation [75]. Among the four LAB strains studied by Muhialdin, Hassan, and Saari [73], the highest antifungal activity of *Lc. mesenteroides* and three *L. plantarum* occurred in different incubation times of 24 h and 48 h, respectively. They highlighted the significance of incubation time, growth stages, and temperature for the production of antifungal compounds. According to them, maximizing the production of inhibitory compounds could be obtained by determining the optimum growth conditions [73].

The inhibitory activities of LAB are strain specific. Selecting the best strain/combination of strains of LAB for biopreservation that would cause the minimum unfavorable changes in the product requires prior experiments. In a study, more than 200 yeast and 200 LAB strains were tested as biopreservatives against fungal growth during the cocoa fermentation process. The most promising candidates among all belonged to only four species of *Lactobacillus fermentum*, *L. plantarum*, *Saccharomyces cerevisiae*, and *Candida ethanolica* [76]. *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 was observed to have strong inhibitory activity against *A. fumigatus*, *P. Roqueforti*, *Aspergillus nidulans*, *Mucor hiemalis*, *Fusarium. graminearum*, *Talaromyces flavus*, *Fusarium poae*, *F. culmorum*, and *Fusarium sporotrichoides*. A weaker activity from the same strain was observed against *Kluyveromyces marxianus*, *Debaryomyces hansenii*, and *S. cerevisiae* while displaying no activity against *Sporobolomyces roseus*, *R. glutinis*, and *Pichia anomala* [71]. Further support for this is another study where the inhibitory percentage of *L. brevis* was stronger than *E. faecium* against *P. chrysogenum* [77].

The selected strains must be adapted or adaptable to the environmental conditions of target food, as well as the production process through which the food is prepared, so that their activity and release of antifungal compounds can be reasonably expected during storage. *L. reuteri* was added to a fermented milk product to inhibit pathogens and spoilage microorganisms. No change in the pH, acidity, soluble solids, color, or rheological aspects of the fermented milk product in the presence of reuterin was observed [78]. Fermentation of an oat-based beverage by *L. plantarum* UFG 121 also best preserved it against *F. culmorum*, causing no differences in terms of some qualitative features as compared to the control [79].

Bacterial metabolite profiles of LAB could sometimes be beneficially modulated, altering their spectrum of antifungal activity. Both the culture medium and the target fungal species determine the antifungal activity of LAB. The quality and quantity of the antifungal metabolites of *Lactobacillus pentosus* ŁOCK 0979 were reported to be dependent on the culture medium compounds. The presence of galactosyl-polyols and gal-erythritol improved the anticandidal properties of *L. pentosus* ŁOCK 0979. The addition of the culture medium of the strain conferred an inhibitory attribute against *Aspergillus brassicicola* and *A. niger.*

4.3. Antifungal Metabolites of LAB

LAB inhibitory compounds are secondary metabolites produced after 48 h of fermentation [70]. Shehata et al. [18] observed that the production of the antifungal metabolites of the LAB strain *Lactobacillus* sp. RM1 was initiated at the growth log phase (12–14 h), reached the highest at the strain stationary phase (24 h), and remained stable. The proposed mechanisms explaining the fungal inhibitory effect of LAB are competition over the available nutrients and space, clogging the pathogen's path through the matrix, and manipulation of the spore membrane, causing viscosity and permeability [80]. Sangmanee and Hongpattarakere [72] revealed that the mechanism of antifungal action of *L. plantarum* K35 supernatant causes damage to the cytoplasmic membrane and cell wall and consequent leakage of cytoplasmic content, the formation of membrane-bound vesicles followed by the destruction of mitochondria and nuclei [72].

Lactic acid, formic acid, acetic acid, caproic acid, and phenyllactic acid (PLA), as organic acids, as well as other metabolites from LAB such as carbon dioxide, hydroxyl fatty acids, hydrogen peroxide, diacetyl, ethanol, reuterin, cyclic dipeptides, protein compounds, reutericyclin, proteinaceous, acetoin, and volatile compounds such as diacetyl are natural antimicrobial and antifungal metabolites produced by LAB [7,58]. Table 2 summarizes the number of LAB studied for their antifungal metabolites, fungal spectrum of activity, and their in situ application in the last 10 years.

Table 2. Selected LAB studied for their antifungal metabolites, fungal spectrum of activity, and their in situ application in the last 10 years.

LAB Isolate	Antifungal Compound	Activity Spectrum	Food Product	Reference
L. pentosus G004 *L. fermentum* Te007 *L. paracasi* D5 *Pediococcus pentosaceus* Te010	Protein-like compounds	*A. niger* and *Aspergillus oryzae*	Bread Tomato Cheese	[81]
L. amylovorus DSM 19280	Acetic acid Lactic acid Hydrocinnamic acid Azelaic acid 4-Hydroxybenzoic acid	*P. expansum*	Cheddar cheese	[62]
L. plantarum LR/14	Antimicrobial peptides AMPs LR14	*A. niger*, *Rhizopus stolonifera*, *M. racemosus* and *P. chrysogenum*	Wheat grain	[82]

Table 2. *Cont.*

LAB Isolate	Antifungal Compound	Activity Spectrum	Food Product	Reference
L. plantarum	Phenolic acids	*F. culmorum*	Barley malt	[75]
	Organic acids	*P. expansum* MUCL2919240	Bread grapes	[83]
L. fermentum, *L. plantarum*	Phenyllactic acid Organic acids	*A. flavus*, *Penicillium citrinum*, *Penicillium griseufulvum*, *A. niger* and *A. fumigatus*	Cocoa beans	[76]
L. reuteri	Reuterin	*P. chrysogenum* and *M. racemosus*	Yogurt	[84]
L. pentosus, *L. plantarum*, *L. brevis*, *Lactobacillus delbrueckii*, *L. fermentum*, *Lactococcus lactis* and *Lc. mesenteroides*	Hydrogen sulphide and lactic acid	*Penicillium oxalicum*, *Fusarium verticillioides* and *A. niger*	Fruits and vegetables	[57]
Lactobaciullus strains	Organic acids	*P. chrysogenum* and *A. favus*	Caciotta cheese	[85]
L. plantarum CECT 749	Gallic, chlorogenic, caffeic and syringic acids	*Fusarium* spp. *Penicillium* spp. and *Aspergillus* spp.	Bread	[86]
Number of LAB strains isolated from Kimchi	Lactic acid and acetic acid	*Cladosporium* sp. YS1, *Neurospora* sp. YS3, and *P. crustosum* YS2	Rice cake	[59]
Leuconostoc spp. *L. reuteri* and *L. buchneri*	Organic acids such as lactic acid, acetic acid and propionic acid	*Aspergillus*, *Eurotium*, *Penicillium*, *Cladosporium* and *Wallemia* spp.	Milk bread rolls	[26]
L. plantarum CH1, *L. paracasei* B20 and *Lc. mesenteroides* L1	Lactic acid and acetic acid	*M. racemosus*, *Penicillium commune*, *Yarrowia lipolytica*, *A. tubingensis*, *A. flavus* and *Paecilomyces*	Sour cream and sourdough bread	[66]

The antifungal metabolites of LAB have the potential to act in synergy. The synergistic effect between the decrease in pH resulted from the production of organic acids and other antifungal metabolites of LAB poses a more efficient final antifungal activity [7]. Peyer et al. [75] demonstrated that there are synergistic effects between organic acids and phenolic acids released by some LAB strains as antifungal metabolites against *F. culmorum*. According to Alex et al. [54], the great synergistic effect between organic acids and antifungal peptides produced by LAB allow the final biopreservation attribute to be influential in bakery products [54].

Antifungal metabolites of LAB have also shown synergy with compounds of other organisms. Ruggirello et al. [76] tested some yeast and LAB strains against six spoilage fungi belonging to *Aspergillus* and *Penicillium* genera during the cocoa fermentation process. The antifungal activity was explained by the synergic production of organic acids (from the LAB) and proteinaceous compounds (from yeasts) [76].

The understanding of the synergy mechanism between antifungal compounds could provide insight in maximizing the impact whilst altering the involved compositions of bacteria or nutrients, eventually leading to actual application in food [75].

4.3.1. Organic Acids

The production of organic acids is believed to determine an LAB strain's mycotoxigenic fungi inhibition properties; the type and quantity of the acids differ from strain to strain [87]. These acids are mainly produced by LAB as a byproduct of acidification process rather than as active synthesis of metabolic compounds aimed at restricting fungi [88].

As the main acid produced by LAB, lactic acid (2-hydroxy propionic acid) is an organic acid widely distributed in nature in two forms of L and D; L lactic acid was recognized as a safe preservative by the FDA [89]. Russo et al. [79] tested the activity of some LAB strains against *Aspergillus*, *Fusarium*, and *Penicillium* and reported that lactic acid was produced at a high concentration during the growth phase as the main metabolic antifungal associated with the low pH. Lactic acid was also identified as the main antifungal compound from *E. faecium*, *L. rhamnosus*, and *L. plantarum* [90]. In the study of Baek et al. [59], the fermentation of rice dough with some LAB isolates from kimchi greatly delayed the growth

of three fungal species in rice cakes. They found lactic acid and acetic acid as the main antifungal substances [59].

The best characterized and most important antimicrobials produced by LAB are lactic acid and acetic acid, which are bioactive in the protonated form at low pH [91]. LAB can produce a variety of compounds at low concentrations and below their minimum inhibitory concentration, which are likely to act synergistically with lactic acid and acetic acid [92,93]. Acetic acid and lactic acid were also proved to display a synergistic antifungal activity in combination; however, due to higher pKa that causes a higher level of dissociation inside the cell, acetic acid has a stronger antifungal activity [94,95]. Loubiere et al. [96] suggested that lactic acid has an inhibitory effect on the metabolism and cell proliferation, which is probably due to the synergistic effect with some of the other side fermentation products such as acetic acid and formic acid [96].

Organic acids of lactic acid, oleic acid, linoleic acid, palmitic acid, 3-PLA, stearic acid, pyroglutamic acid, and 5-oxo-2-pyrrolidine-carboxylic acid were detected as antifungal compounds from *L. plantarum* K35 inhibiting the growth and aflatoxin production of *A. flavus* and *A. parasiticus* [72]. Some other carboxylic acids including benzoic, vanillic, azelaic acid, hydrocinnamic acid, and hydroxy benzoic acid were isolated as antifungal compounds from mediums of *Weissella cibaria* PS2 and three *Lactobacillus* species [97]. Hydrocinnamic acid, azelaic acid, vanillic, p-couramic, and 4-hydroxy benzoic acid were also reported from *L. reuteri* eep1 with antifungal activity [98].

The mechanism of inhibitory activity of organic acids in the growth and activity of many pathogenic and putrefactive bacteria and fungi is attributed to creating an acidic environment and reducing the pH to below the metabolic inhibition and growth range [94]. Loubiere et al. [96] suggested that the inhibitory effect of lactic acid on the metabolism and cell proliferation is probably due to the increase in osmotic pressure of the medium. Organic acids alter plasma membrane permeability and electrochemical, killing the microorganism [80]. In other words, organic acids diffuse to the fungi through the membrane and degrade the cells, thereby releasing hydrogen ions and causing a decrease in pH [99].

For organic acids to penetrate the cell wall, they must be turned to an undissociated form. The pKa of lactic, acetic, caproic acid, and 3-phenyl-L-lactic is 3.8, 4.7, 4.9, and 3.5, respectively [99]. Therefore, in an acidic environment, they will act more efficiently in inhibiting fungi. This fact was proven in the study of Cortés-Zavaleta et al. [7], where they tested the fungal inhibition of the cell-free supernatant of 13 LAB strains against four food-spoilage fungi. The results demonstrated that the inhibition properties dropped when the pH was raised to 6.5 [7].

The production of organic acids confers extra inhibition properties to LAB by activating other antifungal compounds triggered as a result of lowering the pH [99]. Furthermore, organic acids often work in synergy with other compounds, which adds to the complexity of LAB antifungal activity [54]. There is also a synergistic effect between several organic acids together produced by LAB as antifungal compounds. The antifungal activity of *L. plantarum* CCFM259 was assessed against *P. roqueforti*. Acetic acid and PLA showed better antifungal activity than other compounds, and their mixture displayed a synergistic effect [67]. The synergistic contribution of acetic acid was reported in the extension of sourdough fermented by two *Lactobacillus* strains [54].

4.3.2. Phenyllactic Acid (PLA)

PLA (2-hydroxy-3-phenyl propionic acid) is another well-studied organic acid with natural antibacterial properties derived from phenylalanine catabolism. PLA possesses a similar metabolic pathway as lactic acid and is metabolized during fermentation by the glycolytic enzyme and lactate dehydrogenase [100].

The composition of the culture medium was reported to play a great role in the quantity of PLA produced by LAB. The addition of 1.5% (*w*/*v*) phenylalanine to MRS medium of *L. reuteri* R29 significantly increased the production of PLA and, consequently, antifungal performance against *F. culmorum* [101]. The fungal inhibitory strength of PLA produced by

LAB are well-established. Lavermicocca et al. [102] reported that a 10-fold-concentrated culture supernatant of *L. plantarum* 21B inhibited *Eurotium*, *Fusarium*, *Penicillium*, *A. monilia*, and *Endomyces*. Under the same conditions, 3 mg mL^{-1} calcium propionate was not effective, while sodium benzoate performed similar to *L. plantarum* 21B. The antifungal activity of *L. plantarum* 21B was attributed to PLA and 4 OHPLA isolated from the supernatant of the bacteria [102]. In a similar study, *L. plantarum* UM55 was found to produce lactic acid, PLA, OHPLA, and indole lactic acid (ILA). The acids were individually tested against *A. flavus*, and among them, PLA showed the strongest effects with the obtained IC90 for the growth inhibition of 11.9 mg mL^{-1} [103]. PLA and 3,5-Di-O-caf- feoylquinic acids were identified as the predominant antifungal compounds in cell-free supernatant of seven LAB isolated from traditional fermented Andean products with inhibitory activity against a few spoiler fungi from *Penicillium* and *Aspergillus* genus [104]. The antifungal compounds of *L. plantarum* against *A. fumigatus* and *R. stolonifera* resembled the structure of 3PLA with the formed ligands [74].

Although promoting the metabolic pathway of PLA is likely to increase the efficiency, the antifungal properties of PLA depend on a synergistic mechanism with other metabolites [101]. Cortés-Zavaleta et al. [7] tested the fungal inhibition of the cell-free supernatant of 13 LAB strains against four food-spoilage fungi. With two exceptions, all other LAB strains produced PLA ranging from 0.021 to 0.275 mM. They concluded that even if PLA cannot be the only inhibitory compound, it very likely performs in synergy with other acidic compounds from LAB [7]. PLA and acetic acid produced from *L. plantarum* CCFM259 exhibited a synergistic inhibitory effect against *P. roqueforti* [67]. A weak synergistic inhibitory effect of PLA was also reported in combination with cyclo (L-Phe–L-Pro) produced from *L. plantarum* against *A. fumigatus* and *P. roqueforti* [105]. Acetic acid, lactic acid, and PLA produced by *L. plantarum* VE56 and *Weissella paramesenteroides* LC11 exhibited synergism against *A. tubingensis*, *A. niger*, *Candida albicans*, and *P. crustosum* [106].

4.3.3. Reuterin

Reuterin (β-hydroxy propionaldehyde) is a low-molecular-weight multi-compound system consisting of 3-HPA hydrate, 3-hydroxypropionaldehyde (3-HPA), 3-HPA dimer, and acrolein produced by the conversion of glycerol [107]. Reuterin is secreted mainly by *L. reuteri*, though some other bacterial species and genera could also secrete it [107]. Reuterin has a wide spectrum of antimicrobial properties against a range of Gram-positive and Gram-negative bacteria, bacterial spores, molds, yeasts, and protozoa [84,94].

The growth conditions and culture medium can alter the content of reuterin produced by LAB. Schaefer et al. [108] reported the optimum conditions for reuterin production from *L. reuteri* 1063 as culturing the cells for 16 h followed by suspension in 5 mL of 250 mM glycerol in distilled water and incubated for 2 h at 37 °C under anaerobic conditions. Another study reported that supplementation with 150 mM glycerol increased the antifungal activity of *L. reuteri* 5529 cultured in WFH medium. The enhanced antifungal activity of *L. reuteri* 5529 was linked to the production of reuterin. In a similar study, glycerol addition to the culture medium of *L. coryniformis* improved reuterin synthesis, consequently having an antifungal effect against yeast cells and fungal spores and conferring inhibition performance against a couple of new fungal strains [109].

The activity of LAB against fungi is mostly limited to antifungal rather than fungicidal. Reuterin, however, apart from antifungal activity, has also presented a fungicidal effect. Purified Reuterin produced by *L. reuteri* ATCC 53608 fungicidal activity by killing 99.9% of the indicator microorganisms at concentrations equal or below 15.6 mM. As an antifungal agent, it was then added to yogurt. In yogurt also, reuterin exhibited an antifungal effect at a concentration of 1.38 mM while a fungicidal effect at 6.9 mM [84].

The mechanism of action of reuterin has been reported to cause oxidative stress to fungal cells. Reuterin exposure *E. coli* increased the expression of genes regulated and expressed in response to periods of oxidative stress. It was determined that the aldehyde group of reuterin binds to thiol groups of small peptides and other molecules, leading

to oxidative stress, which is hypothesized as the mechanism of inhibition [108]. Another proposed inhibition mechanism of reuterin is through the suppression of ribonuclease activity, which is the main enzyme mediating the biosynthesis of DNA [110], as cited in [68]. More recently, acrolein was reported as the main component conferring antimicrobial activity to reuterin [107].

Reuterin could be a potentially promising candidate as a food biopreservative since in vitro studies using human liver microsomes demonstrated that reuterin does not present the possibility of displaying drug interactions [63]. *P. expansum* was inhibited at concentrations of above 10 mM reuterin produced by *L. reuteri* ATCC 53608 [78]. The addition of *L. reuteri* INIA P572 with glycerol to semi-hard ewe milk cheese resulted in a lower level of 2-heptanone in cheese, which was attributed to the activity of reuterin in mold inhibition [111]. Reuterin was also found to be responsible for the antifungal performance of three *Lactobacillus* and one *Leuconostoc* strains applied in pound cake and milk bread rolls [26].

4.3.4. Peptides and Cyclic Peptides

The antimicrobial peptides are chains of 5–100 amino acid attached through peptide bonds with natural origin (held together through peptide bonds [112]. Protease enzyme treatment is usually employed to determine the peptide nature of the active compounds. The treatment of *Lactobacillus fermentum* CRL 251 supernatant with trypsin, proteinase K, and pepsin decreased the antifungal activity by 50, 4, and 3%, respectively. Further ultrafiltration analysis attributed the activity to smaller fraction of peptides (<10 kDa) [113]. In the study of Magnusson and Schnürer [71], *L. coryniformis* subsp. *coryniformis* presented a strong inhibitory activity against a number of fungi and yeast strains. The activity was attributed to the production of small (3 kDa) and heat-stable proteinaceous antifungal compounds demonstrated by the alteration of activity through the treatment with proteinase K, trypsin, and pepsin [71]. A group of peptides was purified and identified from cell-free supernatants of *L. plantarum* exhibiting inhibitory activity against *A. parasiticus* and *P. expansum* by 58% and 73%, respectively [112]. A total of 37 peptides were identified in the fraction of cell-free supernatant of *L. plantarum* TE10. Treatment of bread with the fraction resulted in slight growth and a fourfold reduction in spore formation of *A. flavus* [73].

Smaller peptides usually possess stronger antifungal activity. Low-molecular-weight peptides (<10 KDa) isolated from the supernatants of four LAB strains represented higher antifungal inhibition against six fungi in comparison with the control supernatant [73].

The mechanism of action of peptides is through binding to lipid bilayers in carpet-like and puncturing channels in it, which impairs the function. They also act through peptide-lipid interaction resulting in phase separation as well as solubilizing the membrane [114].

Low-molecular-weight peptides with high heat-stability from LAB have high potential for replacing chemical preservatives commonly used in the bakery [73]. In the study of Muhialdin, Hassan, and Saari [73], they simulated the maximum heat process of food in manufacturing (121 °C for 60 min) and exposed the supernatants of four LAB strains with antifungal activity to it. The nature of the bioactive substances secreted by bacteria was found to determine whether the activity is heat sensitive and to what extent [73].

Cyclic peptides are composed of polypeptide chains linked covalently in a circular manner. The circular structure is formed either by binding either ends of the peptide chain through an amide bond, or by lactone, thioether, ether, or disulfide bonds [115]. The cyclic dipeptide properties as antifungal agents produced by LAB have been shown in several studies and reviews. *L. plantarum* CM8, *Weissella confusa* I5, *P. pentosaceus* R47, and *W. cibaria* R16 presented inhibitory activity against *P. notatum*. Concentrated supernatants were heated to 80 °C for 1 h followed by an autoclavation step (121 °C for 15 min). No significant influence of heat was observed in the activity of the supernatants. Protease sensitivity properties of the activity implied that the bioactive substances most likely have a proteinaceous nature, perhaps (cyclic) peptides [70].

Cyclo peptide (glycyl-L-leucyl), as a compound that delays the growth of fungi *Fusarium avenaceum*, was isolated from *L. plantarum* [116]. Magnusson [109] also reported the secretion of cyclic dipeptides by *P. pentosaceus* (MiLAB 024), *L. plantarum* (MiLAB 006), and *Lactobacillus sakei* (MiLAB 091). The growth condition of the LAB strain was found to be influential in the quantity of the cyclic peptides released by them. The results obtained by Ryan et al. [88] revealed that acidification of dough fermented by *L. plantarum* FST significantly increased the quantity of cis-cyclo (LPhe-L-Pro) and cis-cyclo (L-Leu-L-Pro) as compared to nonacidified dough [88].

The mechanism of action of antimicrobial cyclic peptides is mainly attributed to the disruption of structural integrity. They target the cell envelope components, causing lysis of the membrane or inhibiting the membrane and/or cell wall biosynthesis [117].

4.3.5. Fatty Acids

Fatty acids are organic acids that possess a carboxyl group (-COOH) and a methyl group (-CH3) at either end [118]. Strong antifungal activities have been reported from fatty acids. Sjogren et al. [119] characterized 3-hydroxydodecanoic acid, 3-hydroxydecanoic acid, 3-3-hydroxy-5-cis-dodecenoic acid, and hydroxytetradecanoic acid from the supernatant of *L. plantarum* MiLAB 14. The hydroxy fatty acids displayed inhibition in the range 10 to >100 μg/mL and were reported to be much more effective than cyclic dipeptides against several molds and yeasts [119].

Fatty acids have been reported in a number of studies to be the main antifungal metabolite preserving foods fermented by LAB. Fermentation of sourdough bread and sour cream by three isolates of LAB of *L. plantarum* CH1, *L. paracasei* B20, and *Lc. mesenteroides* L1 delayed fungal growth in the final food. The main produced compounds were detected to be DL-hydroxyphenyl, 3, 3-(4-hydroxyphenyl) propionic, 4-dihydroxyhydrocinnamic, and 3-(4-hydroxy-3-methoxyphenyl) propanoic acids [66]. Black et al. [69] reported that *L. hammesii* converts linoleic acid to antifungal $C_{18:1}$ monohydroxy fatty acids. Further supplementation of linoleic acid strengthened the antifungal activity of *L. hammesii*. Hydroxylated fatty acids synthesized by the strain were found to be responsible for the extended shelf life of sourdough fermented with *L. hammesii* and the inhibition of *A. niger* and *P. roqueforti* in the bread prepared by that [69].

Little knowledge of the antifungal mechanisms of fatty acids is available so far; however, some pathways have been proposed. Detergent-like properties of the fatty acids affect the structure of cell membranes of the cells, leading to death [119]. Antifungal fatty acids disintegrate lipid bilayers of the membranes and, consequently, cause destruction of the membrane integrity, leading to the disintegration of cells and release of intracellular proteins and electrolytes [120]. Other targets of fatty acids include protein synthesis, which may be inhibited by myristic acid analogues, fatty acid metabolism, as well as topoisomerase activity, which may be inhibited by, amongst others, acetylenic fatty acids [121].

The antifungal activity of fatty acid highly depends on the structure. In the study of Black et al. [69], unsaturated monohydroxy fatty acids were antifungally active; saturated hydroxy fatty acids and unsaturated fatty acids of oleic and stearic acids, however, did not exhibit any activity. This implies the fact that for the fatty acid to function as an antifungal agent, at least one double bond as well as one hydroxyl group along a C18 aliphatic chain should be present in the structure [69].

Pathogenic fungi are less likely to become resistant to antifungal fatty acids [121]. Other antifungal compounds targeting the membrane of fungi are more susceptible to pathogen resistance, which shortens their lifespans. However, as these substances could present synergism with antifungal fatty acids, they could alternatively provide prolonged usage, reducing the required quantity of the antifungal substances [120]. An example of the synergism of fatty acids with other compounds was provided by the study of Ndagano et al. (2011). They observed that 3-hydroxylated produced by *L. plantarum* VE56 and *W. paramesenteroides* LC11 acts in synergy with other bacterial compounds secreted by the bacteria inhibiting *A. niger*, *A. tubingensis*, *C. albicans*, and *P. crustosum* [106].

5. Conclusions

Fungal growth and consequent mycotoxin release in food and feed threaten human health, which might even, in acute cases, lead to death. Addressing the consumer health concern as well as the potential negative risk of using synthetic preservatives, the substitution of LAB as a green preservative could be an alternative due to their safety, health-giving benefits, and preservation properties. LAB release antifungal metabolites against fungal species, which in many cases work in strong synergy.

The application of LAB species with antifungal properties in food can reduce the occurrence of fungal spoilage and toxicity, consequently improving its shelf life as well as causing a reduction in mycotoxins. However, case investigation is required to be carried out individually for each food candidate since the presence of LAB in food can exclusively affect its physiochemical and organoleptic properties, which may or not be desirable. On the other hand, the major population of fungi contaminating a particular food should be regarded in selecting the best LAB/combinations of LAB planned for inhibiting fungal growth in the food. The reason for that is the fact that the antifungal properties of LAB are fungal strain-specific, meaning that an LAB strain might be strongly active against a fungal strain while not causing much disturbance in the viability of another strain.

Almost all antifungal metabolites of LAB present synergy with at least one other component. This fact counts as an advantage in employing LAB as antifungal bacteria in a way to group main producers of synergic components together, thereby maximizing the final activity. The composition of the medium has also been demonstrated to be a significant factor stimulating/raising the release of antifungal compounds by LAB. Therefore, the food nutrient composition is another item to take into account when selecting LAB strains to inhibit fungal growth/mycotoxin control in food. If the formula of the food allows, supplementation with additives along the LAB strains could be a desired alternative, e.g., the addition of phenylalanine along with *L. reuteri* to food in order to increase PLA release.

For an advance in academic studies, enhancement in protection and the safety of products by LAB as probiotics and biopreservatives could be pursued. For food industrial researchers as well, the isolation, formulation, and industrialization of LAB antifungal bioactive metabolites could be of interest.

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Characterization of Bacteriocin Produced by Lactic Acid Bacteria Isolated from Solid Waste of Soymilk production

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Abstract. Biopreservation of food using bacteriocin from lactic acid bacteria (LAB) was an innovative breakthrough. Lactic acid bacteria can protect against food spoilage and pathogen bacteria by producing bacteriocin. The purpose of this study was to characterize the bacteriocin produced by LAB isolated from solid waste of soymilk that had probiotics properties. The LAB having antibacterial activities was evaluated their growth, and identified by using 16S rRNA gene sequence analysis. Its bacteriocin activities was tested on various pHs (2, 3, 4, 5, 6, 7, 8, and 9) and temperature (60-100 ° C). Its activities was evaluated againts pathogenic bacteria (Staphylococcus aureus ATCC 25923 and Listeria. monocytogenes CFSAN004330), enzymes (trypsin, catalase and protease-K), and antibiotics (penicillin and ampicillin). The results showed that LAB A23.4 isolates, which had 16S rRNA gene sequence were L. plantarum strain TMW 1.1623. Its Bacteriocin had antimicrobial activity against S. aureus ATCC 25923 and L. monocytogenes CFSAN004330 at pH 2-7, at temperatures of 60, 70, 80, 90, 100 ° C for 60 minutes and lysed by the enzymes trypsin and protease-K. Bacteriosin activity was stronger than that of the antibiotics of penicillin and ampicillin against S. aureus and L. monocytogenes. The inhibition zone of supernatant bacteriocin was 10 and 20 mm for S. aureus and L. monocytogenes. On the other hand, penicillin and ampicillin inhibition zones were 0 and 3 mm, respectively. From these results, it can be concluded that the antimicrobial produced by L. plantarum strain TMW 1.1623 was a bacteriocin used as food preservation that its processing using relatively wide range temperature (60-100) with pH 2-7.

1. Introduction

Food is a material that is very easy to contamination by spoilage microorganisms because it contains nutrients that are needed by these microorganisms for its growth. The amount of microorganism contamination in food products can affect the quality and shelf life. One way to reduce the occurrence of contamination by microorganisms is to add preservatives.

Bacteriocin is an alternative bio preservative agent that can be used as a food preservative. Bacteriocin is a protein compound produced by lactic acid bacteria [1] that has bactericidal properties against Gram-positive and Gram-negative bacteria. It is very beneficial for the food industry because its activity can inhibit the growth of disease-carrying bacteria that are usually present in food [2]. Some

LAB species produced chemical compounds that had microbial activities such as bacteriocin and hydrogen peroxide [3].

Research on antimicrobial compounds from LAB had been carried out including, research by Ref [4] about bacteriosin produced by Lactococcus lactis subsp. lactis strain isolated from traditional food fermentation. The antimicrobial activity of bacteriocin produced by Lactobacillus Sp. isolated from traditionally produced milk was observed by Ref. [5]. Purification and characterization of the F1 bacteriocin, a bacteriocin produced from the L. paracasai subsp. from Tibetan kefir, China [6]. The results showed that F1 bacteriocin was the first bacteriocin produced by L. *paracasei* subsp. and this bacteriocin is potentially used in the food industry.

Lactobacillus isolated from solid waste of soymilk processing [7] was a potential source of isolates for producing antibacterial, and they also have probiotics properties8. LAB, which has probiotic properties and can also produce bacteriocin, is a characteristic of LAB that is very well used in food processing. This LAB can act as a probiotic and a food preservative that can extend the shelf life of the food. The objectives of this study were to characterize bacteriocin produce Lactobacillus spp having probiotics properties that were isolated from solid waste of soy milk production,

2. Materials and Methods

2.1. Materials

Materials used were Lactobacillus isolated from solid waste of soymilk production, indicator bacteria (Escherichia coli O167: H7, Staphylococcus aureus ATCC25923, and Listeria monocytogenes CFSAN004330), antibiotics (Penicillin, Ampicillin and Kanamycin) disks, EDTA, Lysozim, Rytomysis, Nuclear Solution -nase solution, Protein precipitation solution, isopropanol, ethanol, Rehydration solution, Primer R and F, TE Buffer, dH2O, Master Mix, Template, Agarose, TAE, and redsafe / gel view staining. (Merck), MRS Broth (Merck), Nutrient Agar (Merck), Nutrient broth (Merck), listeria broth (Merck), Buffer Pepton Water, glycerol, aquades, alcohol spritus, HCl 6M, NaOH 6M,

The equipment used is cool box, anaerobic jar, thermometer, pH meter, petri dish, ose needle, incubator (Infors HT-ecotron), PCR (Biometrasentrifus Sartorius Sigma), Biodoc Analyze (Biometra), Electrophoresis (Mupid Exu), measuring cup , analytical scales, erlemeyers, drop pipettes, bunsen lamps, test tubes, measuring cups, hocky stics, cotton buds, beaker glass, autoclaves, and micropipettes.

2.2. Research Methods

2.2.1. Cell-free supernatant antimicrobial activity

This method referred to diffusion method 9 as much as 50 µl of the supernatant was put into the wells that had been provided. Petri dishes and their contents were placed for two hours in a refrigerator so that the supernatant seeps into the agar medium. Then incubated at 37°C for 24 hours. The clear zone formed indicates the inhibition of the growth of test bacteria by the supernatant. Clear zone diameters were measured using a caliper three times with different positions and averaged.

2.2.2. Determination of Lactic Acid Bacteria Growth

Lactic acid bacteria isolate was inoculated in 1 ose. It was incubated in 9 ml MRS-B media for 48 hours at 37 ° C. Bacterial growth was observed in optical density / OD values at 0 and 3 hours and followed every 6 hours for up to 48 hours. It used the turbidimetry method with 650 nm wavelengths by using a visible UV spectrometer to a constant absorbance value indicating the LAB growth has reached the stationary phase. If the value of absorbance reading is above 1, it was necessary to dilute it with sterile MRS-B10.

2.2.3. Identification Microorganism Using 16S rRNA

The species of selected LAB were identified based on 16S rRNA gene sequence analysis8. Genomic DNA from the isolate was extracted using kit Presto™ Mini gDNA Bacteria. The DNA gene was amplified by PCR using the universal primers 27 F (5'-GAGTTTGATCCTGGCTAG-3'), 1525 R (5'-

AGAAAGGAGGTGATCCAGCC-3'). The conditions of PCR amplification were programmed as follows: Initial denaturation for 5 min at 95°C. 40 denaturation cycles at 94°C for 45 sec/each, 1 min annealing time at 56°C, 1 min and 30 sec for the extension at 72°C, and 7 min for a final extension at 72°C. The amplification products were analyzed by electrophoresis using 1% (w/v) agarose gel and visualized. The electrophoresis gel was stained with 5 µg / ml ethidium bromide solution by immersion, then visualized over UV light (WiseUV WUV-M20) and photographed with a digital camera (Olympus SP-500 UZ). The 16S rRNA sequence was compared with the sequences available in the nucleotide database using the BLAST (Basic Local Alignment Search Tool) at the NCBI server.

2.2.4. Antibacterial Activity of Bacteriocin against Pathogens and Antibiotics

One ml of culture was incubated for 24 hours in 9 ml of MRS Broth at 37 ° C. Then, centrifuged at 14,000 rpm for 5 minutes. The supernatant was filtered with a 0.22 µl membrane filter. Cell-free supernatant is adjusted to pH 6.5 with 1N NaOH, to eliminate the inhibitory effect due to the presence of organic acids11. Pathogenic bacteria are grown aerobically at 37 ° C for 24 hours. Then a 0.2% pathogenic bacterial culture was inserted into 20 ml NA at 50 ° C. After solidifying, the well is 4 mm in size, using a cork borer. Fifty ml supernatant put in each well, and an antibiotic disk was placed on the surface of the media, allowed to stand for 15-20 minutes. Furthermore, it was incubated for 24 hours at 37 ° C under aerobic conditions. Then the inhibition zone is measured using a caliper.

2.2.5. Antibacterial Activity of Bacteriocin at Different Degrees of Acidity

Ten ml of MRS Broth were prepared with pH 2, 3, 4, 5, 6, 7, 8, and 9 respectively using 1 N HCl or 1 N NaOH and autoclaved. After that, it was incubated for 2 hours at 37 ° C, and each pH was adjusted back to pH 6.0. The inhibitory activity measured by all treatments was determined by the agar diffusion method as described above, and S. aureus and L. monocytogenes as indicators of bacteria (Chen, et al. 2014). Each was carried out three times, and repeated standard deviations were measured.

2.2.6. Antibacterial Stability Bacteriocin against Heat

Cell-free supernatant was treated at 40, 60, 80, and 100 ° C for 60 minutes compared to controls (without heat treatment). Then tested by diffusion method so that it uses bacterial indicators12. The method used in this research was the descriptive method. The temperature treatments are: (a). 40 ° C, (B). 60 ° C, (C). 80 ° C, and (D). 100 ° C for 30 minutes. The activity of barriers measured by all treatments was determined by the agar diffusion method as described above and S. aureus and L. monocytogenes as indicators of bacteria. Each was done three times, and standard deviations were measured.

2.2.7. Bacteriocin Extraction and Purification

LAB isolates that have the best resistance to pathogenic bacteria based on temperature, followed by bacteriocin purification13. LAB isolates were grown in 60 ml MRSB. Then centrifuged at a speed of 13,000 rpm for 10 minutes at 4 ° C. The supernatant was neutralized with 1 N NaOH to pH 6.5 and filtered using a 0.22 µm membrane filter. Ammonium sulfate (30%, 60%, and 90% gradually) is poured into 200 ml supernatant and stirred until the ammonium sulfate dissolves. The solution was stored at 4 ° C for 3 × 6 hours. Then the solution was centrifuged at 4000 rpm for 30 minutes at 4 ° C. The precipitate formed was dissolved in 9 ml of citrate phosphate buffer (50 mM: pH 5.0). Then bacteriocin activity was measured in two stages: Cell-free supernatant and bacteriocin extract after precipitation with Ammonium Sulfate

2.2.8. Effect of Enzymes on the Pathogenic Bacterial Activity

Testing was done by the agar diffusion method (Djordjevic et al. 2015; Muse and Hartel 2004). The 200 µl supernatant from LAB was dissolved in 20 ml each of the enzymes dissolved protease-K (pH 7), catalase (pH 7), and trypsin (pH 7). These enzymes were dissolved in NaOH or phosphate buffer pH 7. Supernatant that has been mixed with this enzyme was incubated for 2 hours at 37 ° C, and followed by heating in boiling water for 5 minutes. Supernatants that are sensitive to enzymes are not clear zones.

2.3. Data Analysis
All experiments were carried out in quadruplicate. Statistical analysis was carried out using Excel software (Windows 2010).

3. Results and Discussion

3.1. Cell-free supernatant antimicrobial activity
Six of the 24 LAB isolates isolated from solid waste from soy milk production had antimicrobial activity on all three indicator bacteria. One of these isolates (A23.4) has the highest antimicrobial activity, which is characterized by the formation of a clear zone greater than 8 mm in each bacterial pathogens. Cell-free supernatant had inhibitory activity against the growth of Escherichia coli 0157: H7, Staphylococcus aureus ATCC 25923, and Listeria monocytogenes CFSAN004330. The diameter of the cell-free supernatant inhibition against pathogenic bacteria could be seen in Figure 1.

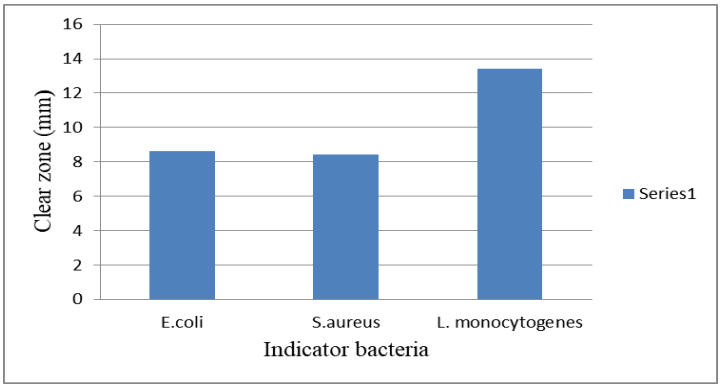

Fig 1. Antimicrobial activity of cell-free supernatant of A23.4 isolate against pathogens, Clear zone diameter does not include wellbore

In Figure 1 the highest antimicrobial activity of cell-free supernatant was found in L. monocytogenes (13.43 ± 0.24 mm). These results illustrate that LAB isolates could inhibit the growth of pathogenic bacteria. Cell-free supernatants had different abilities to inhibit the growth of pathogenic bacteria and produce antimicrobials. Secondary metabolites of LAB were produced extracellularly so that the supernatant was the result of the separation between the cell and the liquid portion containing the secondary metabolite. The secondary metabolite products include organic acids, hydrogen peroxide, diacetyl, and bacteriocin. The LAB secondary metabolites can function as self-defense [15] .

3.2. Determination of Lactic Acid Bacteria Growth
Based on the selection results to obtain LAB having the potential as a probiotic in previous studies16, the A.23.4 isolate was chosen. In Figure 2. it can be seen that in the initial incubation stage from 0 to 3 hours, there was a lag phase. In this phase, LAB was in an adaptation period where growth was very slow, or at this time, there was no significant bacterial growth. The results obtained were almost the same as the results of research on determining the optimum incubation time of LAB to produce bacteriocin, where the first 2 hours of incubation period did not occur significantly LAB growth [17]. The LAB growth could be observed through an increase in OD values, which increases OD values (Figure 2) in line with the increase in turbidity of the suspension, which indicates that there is an increase in bacterial multiplication.

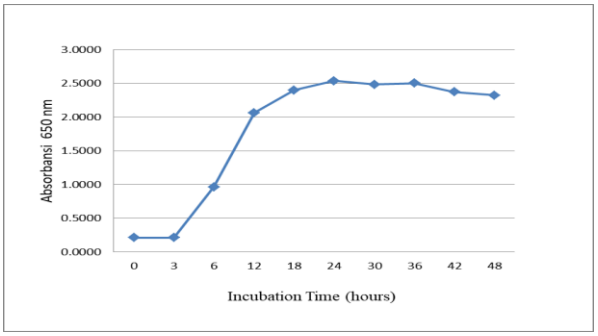

Fig 2. Growth curves of A23.4 LAB isolate in MRS broth media

The A23.4 LAB isolates entered the period of suspension at the 24 to 36 hours from the time of incubation. At this time in the medium of nutrient availability had decreased, and LABs began to produce secondary metabolic, namely bacteriocin, which was extracellular secondary metabolic.

3.3. Identification of Lactic Acid Bacteria with 16S-RNA

Isolation and PCR amplification of 16S-RNA genes to the genome DNA isolate A.23.4 showed in Figure 3. These results could be seen from the visualization of electrophoresis, which was a 1444 bp DNA tape using 27F primers using 27F primers Primer R (GTTTACCTT GTTACGACTT) and F (AGAGTTTGATCCTGGCTCAG). The DNA sequencing results of A.23.4 isolates were analyzed using the BLAST software program on the NCBI website (http://www.ncbi.nlm.nih.gov).

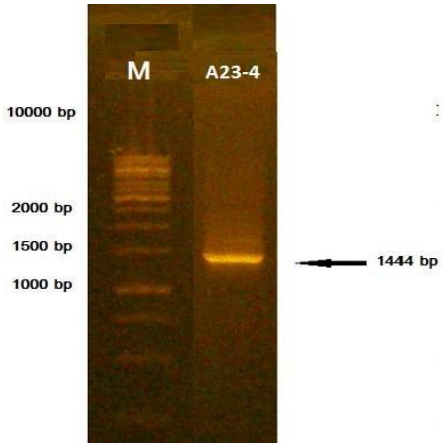

Fig 3. Electrophoresis results of agarose gel genome DNA isolate of Lactic Acid Bacteria (A.23.4), M = marker

The results of genome base sequence analysis with 16S-RNA showed that isolate A.23.4 had similarity (100%) with sequence nucleotide L. Plantarum strain TMW 1.1623. The phylogenetics was shown in Figure 4.

Based on the results of the BLAST analysis identified BAL species from soybean milk solid waste production in the form of soybean pulp with code A.23.4 have 100% similarity. L. Plantarum strain TMW 1.1623, and this is also from phylogenetic trees (Figure 5). L. Plantarum bacteria were mostly found by previous researchers on spontaneously fermented vegetable ingredients such as spontaneous fermented green olives [18], from Amasi, a Zimbabwean fermented milk product [19], and Nigerian fermented product [20]. Besides, L. Plantarum isolated from dairy products also has activity as a probiotic and antimicrobial as reported by Ref. [21].

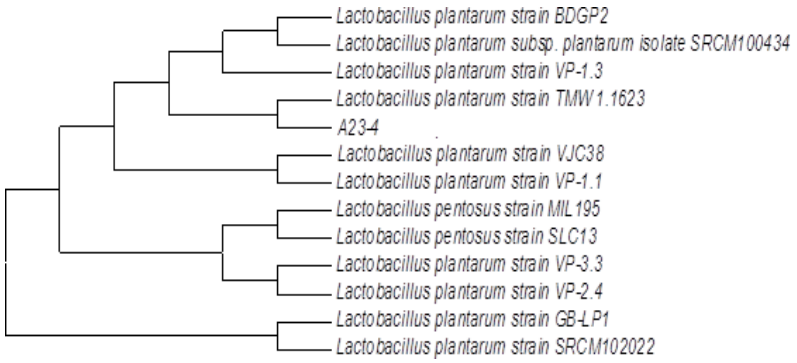

Fig 4. Phylogenetic tree of Lactobacillus plantarum strain TMW 1.1623

3.4. Antibacterial Activity of Bacteriocin at Different Degrees of Acidity

The bacteriocin characterization was carried out by testing its antimicrobial activity against pathogenic bacteria. The bacteriocin antibacterial activity of Lactobacillus plantarum strain TMW 1.1623 was evaluated in different pH environments, ie 2, 3, 4, 5, 6, 7, 8, and 9. The results were shown in Figure 7. where an increase in the diameter of inhibitory zones against L. monocytogenes at pH 2 and 3 and a decrease in inhibition zone diameter to pH 7. However, at pH 8 and 9, no inhibition zone was formed. On the contrary, this antimicrobial activity against S. aureus shown that the inhibition zone was up to pH 7, but the diameter of the inhibition zone against S. aureus was lower than the inhibitory zone against L. monocytogenes. The formation of inhibitory zones at relatively high at pH 8 and 9. It was due to the bacteriocin, which was a secondary metabolic of Lactobacillus plantarum strain TMW 1.1623. It was a protein molecule with a relatively low molecular weight, so an extreme increase in pH resulted in protein denaturation.

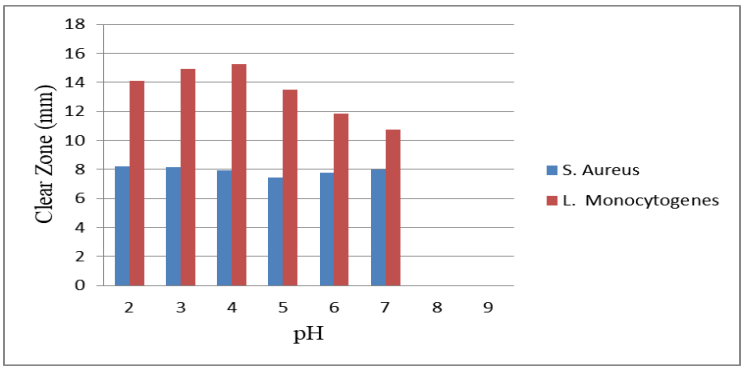

Fig 5. Effect of pH on the bacteriocin antimicrobial activity of L. plantarum strain TMW 1.1623

Denaturation of this protein caused physical changes in its structure so that its biological function becomes damaged. Because these bacteriocins did not exhibit antimicrobial activity, not found a clear zone. It was a condition, due to physical damage caused by protein denaturation. This situation was expressed by Ref [22] that there was a strong intra-molecular electrostatic interaction that causes dissociation between amino acids and carboxyl groups so that protein denaturation occurs in alkaline conditions.

3.5. Antibacterial stability of bacteriocin against heat

The sensitivity test results of bacteriocin extract could be seen in Figure 7. The results showed that an increased in temperature from 60-100 ° C decreased the bacteriocin antimicrobial activity, which was

shown to decrease the diameter of the clear zone (Figure 10) both against S. aureus or L. monositogenes. Bacteriocin was a short-chain peptide compound that was easily damaged by heat, according to Ref [23], bacteriocin would be damaged due to heat treatment which would adversely affect its bioactive activity.

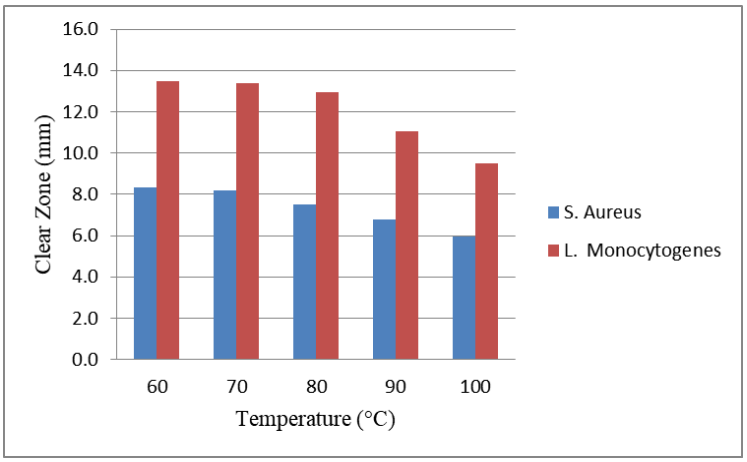

Fig 6. Heat sensitivity to the antimicrobial, antifungal bacteriocin of L. Plantarum strain TMW 1.1623

In the results of this study can be seen in Figure 7, that the activity of bacteriocin extract at treatment up to 100 °C for 60 minutes against L. monocytogenes bacteria still shows antimicrobial activity. It appears that the formation of a clear zone at a temperature of 100 ° C whose diameters are 6.6 and 9.5 mm respectively for S. aureus and L. monositogenes. Whereas the antimicrobial activity decreases with an increase in temperature of more than 80 ° C. This is because bacteriocin was a protein compound that was denatured due to high temperatures. The protein could be easily denatured if exposed to relatively high temperatures (> 80 ° C) [24].

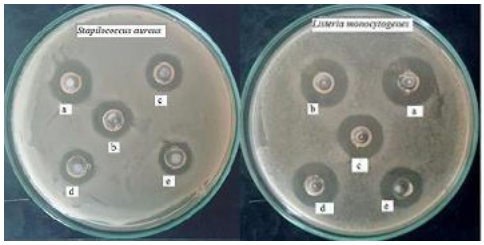

Fig 7. The sensitivity of bacteriocin extract at various temperatures (A = 60 ° C, B = 70 ° C, C = 90 ° C, D = 90, and E = 100 ° C)

In this study, it was seen that the antimicrobial activity of bacteriocin extract was higher in L. monocytogenes more than in S. aureus pathogen. The bacteriocin activity indicated by formating a clear zone at 100 C was 10.20 mm. The bacterial activity of the bacteriocin extract produced by L. plantarum had the potential to be used as a bio preservative for food processing. This result was in line with the conclusions of Ref. [25] which produces bacteriocin from L. plantarum 2C12 which had an antimicrobial activity that can inhibit the growth of pathogenic bacteria such as E. coli, S. aureus, and Salmonella typhimurium

3.6. Bacteriocin supernatant extraction and purification

The bacteriocin supernatant activity of L. Plantarum strain TMW 1.1623 at the ammonium sulfate concentration stage for precipitation was 30, 60 and 90% compared to the control antimicrobial activity (supernatant free cell). Pellets in the form of bacteriocin were evaluated for their antimicrobial activity against pathogens (L. Plantarum strain TMW 1.1623). Figure 8 show the antimicrobial activity of

bacteriocin against S. aureus and L. monocytogenes, where the higher the fraction of the ammonium sulfate usage, the better the purity level. This bacteriocin had better antimicrobial activity, which was indicated by existing a clear zone.

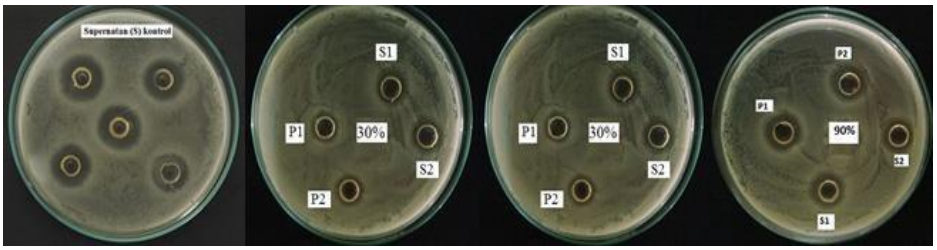

Fig 8. Bacteriocin antimicrobial activity after precipitation with ammonium sulfate (P = bacteriocin pellet, S = supernatant

3.7. Effect of Enzymes on the Pathogenic Bacterial Activity

The bacteriocin was evaluated using several enzymes (protease K, trypsin, and catalase), and pathogenic bacteria. The results of these three enzymes can be seen in Table 1, where the bacteriocin extract added by the protease K enzyme and trypsin loses its antimicrobial activity against S. aureus and L. monocytogenes to various enzymes. This was indicated by the absence of clear zones in the two pathogens. The absence of this clear zone was caused by bacteriocin, which was a peptide compound [26] whose low molecular weight protein was hydrolyzed by the enzyme protease K and trypsin. These data obtained were similar to the results of study of Ref. [20], where evaluated the bacteriocin produced by L. plantarum F1. The results showed that antimicrobial activity was lost or unstable after bacteriocin treated with proteolytic enzymes. The same thing was also obtained from the results of a study of the bacteriocin activity of L. plantarum derived from cow's milk [10], apparently, the bacteriocin was very sensitive to proteolytic enzymes, such as trypsin, pepsin, and proteinase K. Bacteriocin in food was be degraded by proteolytic enzymes in the digestive tract [27].

Based on the results, the enzyme could inactivate bacteriocidal activity in inhibiting bacterial pathogens. Conversely, without the addition of enzymes to the bacteriocin extract the anti-microbe activity remained active, this was shown in control (Table 1) there were inhibitory zones in S. aureus and L. monocytogenes, respectively 6.27 and 11.33 mm.

Table 1. Effect of enzymes on the bacteriocin extract activity of Lactobacillus plantarum strain TMW 1.1623

Treatments	**Clear Zone (mm)**	
	Stapilococcus aureus	***Listeria monocytogenes***
Control	6.27±0.12	11.33±0.74
Protease K	-	-
Trypsin	-	-
Catalase	5.33±0.35	10.10±0.46

The effect of enzyme catalase on bacteriocin extract from L. plantarum strain TMW 1.1623 continued to provide an anti-microbial activity againts S. aureus ATCC 25923 and L. monocytogenes CFSAN004330. Inhibitory zones formed on the two pathogens were approximately 5.33 and 10.10 mm respectively against S. aureus and L. monocytogenes. The addition of the enzyme catalase to bacteriocin extract from L. plantarum strain TMW 1.1623 did not affect the anti-microbial activity against pathogens S. aureus and L. monocytogenes. Inhibition zones formed on two pathogens were respectively 5.33 and 10.10 mm against S. aureus and L. monocytogenes. The addition of the enzyme catalase to bacteriocin extract from L. plantarum strain TMW 1.1623 did not affect the anti-microbial activity against pathogens

S. aureus and L. monocytogenes. Inhibition zones formed on two pathogens were respectively 5.33 and 10.10 mm against S. aureus and L. monocytogenes. The bacteriocin still had antimicrobial activity, even though the enzyme catalase was added to the media. This also shows that the bacteriocin activity is not influenced by hydrogen peroxide12. Bacteriocin from LAB was sensitive to proteases, but resistant to catalase [28].

4. Conclusion

The bacteriocin supernatant produced by Lactobacillus plantarum strain TMW 1.1623 has antimicrobial activity in the range of pH 2-7 and a temperature of 60-100ºC against Staphylococcus aureus ATCC 25923 and Listeria monocytogenes CFSAN004330. This bacteriocin is sensitive to protease K, trypsin, but not sensitive to the enzyme catalase.

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ARTICLES FOR FACULTY MEMBERS

EFFECT OF LACTIC ACID BACTERIA AS BIO-PRESERVATION AGAINST SPOILAGE FUNGI FROM PAPAYA FRUIT

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Combined transcriptomics and metabolomics analysis reveals the antifungal activity and mechanism of *Enterococcus faecium* cell-free supernatant against *Alternaria alternata*

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ABSTRACT

Certain lactic acid bacteria have been utilized as bacteriostatic agents in the context of fruit and vegetable spoilage fungi. Although *Enterococcus faecium* is a member of the lactic acid bacteria group, its application in the prevention of post-harvest fruit spoilage remains largely unexplored. We investigated the activity of *E. faecium* cell-free supernatant (CFS) against fruit and vegetable spoilage fungi and probed the possible fungistatic mechanisms involved. The *in vitro* results showed that CFS possessed significant antifungal activity against *Alternaria alternata* with a MIC of 20%, which resulted in distorted rupture of *A. alternata* cell membranes and leakage of cellular components in a dose-dependent manner. In addition, transcriptomics and metabolomics results showed that CFS could also inhibit the growth of *A. alternata* by altering energy metabolism, impairing intracellular antioxidant function, reducing intracellular hormone biosynthesis, and slowing down or stopping asymmetric cell division. In summary, *E. faecium* CFS has been shown to effectively inhibit *A. alternata*.

1. Introduction

Biological contamination not only poses a serious threat to human health (Thery, Lynch, Zannini, & Arendt, 2020), but also causes severe damage to the fruit and vegetable industry. According to statistics (Yu et al., 2020), the damage caused by postharvest fruit pathogens can be up to 20%–40% in developing countries where inadequate refrigeration and transportation facilities exist. In these regions, fungal spoilage can cause cosmetic defects as well as internal nutrient depletion in fruits and vegetables, and it is the leading cause of economic losses in the post-harvest stage (Yu et al., 2020). The *Alternaria* genus is a widespread destructive fungus with high adaptability (Guo, Qiao, Ji, Wang, & Zhu, 2020), exhibiting rapid growth and reproduction, and possessing a high spore-forming ability. It can infect many vegetables and fruits and cause decay during storage and transportation. *A. alternata* is a paramount mycotoxin-producing species among *Alternaria* spp. (Estiarte, Lawrence, Sanchis, Ramos, & Crespo-Sempere, 2016), which produces mycotoxins, mainly including altertoxins, alternariol monomethyl ether, altenuene, and tenuazonic acid (Kong et al., 2023a); these mycotoxins are mutagenic and carcinogenic to animals and humans, and have a tremendous negative impact on human health (Pereira et al., 2024; Wang, Jiang, Wang, & Feng, 2017). Therefore, controlling *A. alternata* on fruit and vegetable products is crucial.

Coupled with the trend towards healthier and non-chemical consumption, the public health and environmental aspects of fruit and vegetable postharvest processing are gradually increasing in importance. As a result, there is an increasing need to explore effective, safe, and sustainable fungal inhibition methods in the fruit and vegetable postharvest field has become increasingly evident. In recent years, many natural antifungal substances, such as essential oils, phenolic acids (Shu et al., 2019; Wang, Fu, et al., 2023) and their derivatives, as well as terpenoids, have been shown to inhibit the growth of *A. alternata* and to control the occurrence of postharvest diseases in fruits and vegetables (Jiang, Wang, Li, & Sun, 2023). However, the practical application of these agents faces notable challenges. Essential oils, for instance, suffer from issues such as poor water solubility, high volatility, and intense odors, while the effectiveness of phenolic acid-based agents is limited by their low water solubility. Moreover, these agents may cause skin irritation and, if used improperly, present a risk of intestinal absorption, which provides additional challenges in practical applications.

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In recent times, omics analysis has emerged as a robust tool in agricultural research, offering deep insights into the interactions between biocontrol agents and pathogens. Transcriptomics and metabolomics, in particular, have been instrumental in elucidating the mechanisms of antifungal activity of various biocontrol agents (Chen, Zhang, Tian, & Li, 2022). Transcriptomic analysis helps in understanding the gene expression changes induced by biocontrol agents in pathogens like *A. alternata*, shedding light on the molecular pathways affected (Bi et al., 2023; Kong et al., 2023b; Yu et al., 2023). Metabolomics complements this by providing a comprehensive profile of metabolic alterations, thereby revealing the biochemical strategies employed by biocontrol agents to inhibit fungal growth (Zhang et al., 2022b). These approaches have significantly enhanced our understanding of the dynamic interactions between biocontrol agents and pathogens, guiding the development of more effective and targeted antifungal strategies.

Enterococcus is a genus within the lactic acid bacteria (LAB) group, which plays a crucial role in the fermentation process of various foods. These bacteria are especially prevalent in the production of certain cheeses and fermented sausages, where they contribute to ripening and aroma development. Certain strains of *E. faecium* are also recognized for their probiotic properties and have been used to improve human and animal health, particularly in the treatment of conditions such as irritable bowel syndrome (Fan, Chen, Yu, Si, & Liu, 2006), diarrhea or antibiotic-associated diarrhea (Underdahl, Torres-Medina, & Dosten, 1982), or for the enhancement of immune parameters or other health-related factors to improve growth performance and well-being (Capcarova et al., 2011). In particular *Enterococcus faecalis* NCIMB 10415 has been approved for use as a feed additive in different production animals, and *Enterococcus faecalis* M157 KACC81148BP has been used for the treatment of periodontal disease and to improve intestinal health (Park, Ha, Lim, Kim, & Yoon, 2021).

Despite the beneficial applications of *E. faecium*, concerns about its uncontrolled use are justified due to the potential for nosocomial infections, antibiotic resistance, and pathogenicity (Arias & Murray, 2008). However, Zhong et al. (2023) have recently provided evidence affirming the safety of specific strains of *E. faecium* used as probiotics. In light of these findings and the dual characteristics of *E. faecium*, meticulous strain selection becomes imperative for its functional application. This study is precisely such an attempt, evaluating the antifungal properties of the cell-free supernatant of *E. faecium*, notably against *A. alternata*, a common spoilage agent in fruits and vegetables. By means of comprehensive transcriptomics and metabolomics analyses, combined with examination of mycelial growth, ultrastructural changes, and membrane integrity, we aim to elucidate the mechanisms underpinning the antifungal activity. Based on the existing safety profile of *E. faecium*, our future work will include empirical evaluations to further verify the safety and efficacy of *E. faecium* in food preservation contexts.

2. Materials and methods

2.1. Strains and culture media

The *E. faecium* utilized in this research were procured from naturally fermented berries. Following isolation, these bacteria were accurately characterized as *E. faecium* strain LY31 through comprehensive 16S rDNA sequencing analysis. This strain, with accession number MT740410.1, is documented in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/nucleotide/MT740410.1). For preservation and subsequent experimental use, the strain was maintained under optimal conditions in our laboratory's culture collection. In parallel, a spoilage fungus was isolated from decayed indigo fruit specimens. This fungal isolate was subjected to rigorous DNA sequencing of its Internal Transcribed Spacer (ITS) region, leading to its identification as *A. alternata*. The sequence data for this fungal species are also available in the NCBI database (https://www.ncbi.nlm.nih.gov/nucleotide/MT498268.1). Cultivation of *E. faecium* strain LY31 was executed in a specialized De Man Rogosa and Sharpe (MRS) medium. *A. alternata* was propagated on Potato Dextrose Agar (PDA), with incubation conducted at a constant temperature of 28 °C to facilitate optimal fungal growth. All reagents and culture media used in this experiment were obtained from Shengze Technology Co., Ltd., located in Harbin, Heilongjiang Province, China.

2.2. Preparation of E. faecium cell-free supernatants

In this study, we employed a modified methodology, as proposed by Arrioja-Breton, Mani-Lopez, Palou, and Lopez-Malo (2020) , to obtain the CFS from cultures of *E. faecium*. Initially, *E. faecium* was cultured until it attained the logarithmic phase of growth, which is typically characterized by a concentration of approximately 10^7 Colony-Forming Units (CFU)/mL, at an incubation temperature of 37 °C. Subsequently, the culture underwent centrifugation at a force of 8000×*g* for a duration of 10 min at a temperature of 4 °C. The resultant supernatant was then meticulously filtered using sterile 0.22 μm polyethersulfone (PES) disposable needle filters. The filtrate obtained from this process was designated as the CFS, which was utilized for subsequent experimental investigations.

2.3. Preparation of A. alternata fungal suspension

A. alternata was inoculated in a dish with PDA and incubated at 28 °C for 5 days. Subsequently, under sterile conditions, the fungal mycelium was transferred to a 100 mL conical flask containing 20 mL of Potato Dextrose Broth (PDB). The culture was incubated in a model HSHZ-A water bath shaker (Shanghai Yuejin Hengyue Co., Ltd., China) until it reached the logarithmic phase of growth. It was then mechanically disrupted with a high-speed homogenizer (Shanghai Blue Kai Technology Co., Ltd., China) at 8000 rpm for 30 s, and then diluted to 6.4×10^6 CFU/mL with PDB.

2.4. In vitro evaluation of the antifungal activity of CFS

The method previously described by Pandey (Pandey, Gupta, Paul, & Tilak, 2020) and Wang (Wang, Fu, et al., 2023) was used with modifications. CFS was diluted with sterilized MRS to a final concentration of 0.625%–60% of the original concentration in successive dilutions, mixed well with PDA cooled to 50 °C, respectively. Then 20 mL of it was poured into Petri dishes, using the medium without CFS as a positive control and the medium with the addition of MRS alone as a negative control. After the medium solidified, 50 μL of fungal suspension was aspirated with a pipette gun, evenly spread on the surface of the medium, and incubated at 28 °C for 72 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of CFS that resulted in a notable decrease in *A. alternata* colony diameter when assessed visually. In contrast, the minimum fungicidal concentration (MFC) referred to the CFS concentration at which no fungal colony growth was observed. The test was with three repetitions of three parallel test samples each time.

2.5. Colony growth assay

PDA medium containing different concentrations of CFS (0, 0.5, 1, and 2 MIC) was poured into Petri dishes, and MRS medium was used as a control. Disks of *A. alternata* with a diameter of 5 mm were inoculated in the center of each Petri dish using the spot-touch method and incubated at 28 °C. Photographs of the colony growth were taken on days 3 and 7, and the colony diameter of each group was measured with a vernier caliper.

2.6. Effect of CFS on cell morphology of A. alternata

Changes in the morphology of *A. alternata* mycelium were examined using previously reported methods with minor modifications (Yahyazadeh, Omidbaigi, Zare, & Taheri, 2008). Briefly, fresh mycelia of *A. alternata* cultured for 3 d were treated with various concentrations of CFS (0.5, 1, and 2 MIC) at 28 °C for 12 h. Untreated mycelium was used as a negative control. Samples were washed three times with 0.1 M phosphate-buffered saline (PBS; pH 7.0) and fixed with 5.4% (v/v) glutaraldehyde for 24 h at 4 °C. The fixative was discarded, and the mycelium was rinsed three times (15 min each) with 0.1 M PBS (pH 7.0). Subsequently, the samples were first dehydrated in different ethanol concentrations (50%, 60%, 70%, 80%, 90%, and 100%) for 15–20 min, respectively. The dehydrated mycelium samples were transferred to a mixture of ethanol and isoamyl acetate (v:v = 1:1) for about 30 min and then to pure isoamyl acetate for about 1 h. Finally, the samples were critical point dried with liquid CO_2, coated with gold-palladium, and observed under a scanning electron microscope (SEM) (SU-8010, Hitachi Ltd., Tokyo, Japan).

2.7. Detecting the conductivity and leakage of intracellular constituents

Relative conductivity was measured with moderate modifications following previous studies (Li et al., 2021). The cultures were incubated in PDB medium on a gyratory shaker (120 rpm, 28 °C) for 3 d, and then fresh mycelium was filtered through gauze and washed three times with sterile water. Subsequently, 0.5 g of new mycelium samples were suspended in 25 mL of PDB medium containing different concentrations of CFS (0.5, 1, and 2 MIC) and incubated on a gyratory shaker (120 rpm, 28 °C). Sterile water was used instead of CFS as a negative control. After 0, 1, 2, 4, 6, 8, 10, and 12 h of incubation, the conductivity of the mycelial suspensions was measured by a conductivity meter (DDSJ-319 L, Shanghai Insa Scientific Instruments Co., Ltd., Shanghai, China). Finally, each treated sample was boiled for 10 min, and the boiling conductivity was determined after the samples were cooled to room temperature. The relative conductivity was determined using the formula:

$$\text{Relative conductivity} = (D1 - D0)/(D2 - D0) \times 100$$

where D0 = initial conductivity (0 h), D1 = conductivity at different time points, and D2 = boiling conductivity.

After incubation for 3, 6, 12, 24, and 48 h, the supernatant was obtained by centrifugation at 6000×*g* for 15 min. Finally, the absorbance values of extracellular proteins and nucleic acids at 260 nm and 280 nm, respectively, were measured using a 96-well UV microplate (Super Max 3100, Shanghai Flash Spectrum Biotechnology Co., Ltd., Shanghai, China).

2.8. Detection of plasma membrane integrity in A. alternata

A. alternata cultured for 72 h were incubated with 0.5, 1, and 2 MIC of CFS at 28 °C on a water bath shaker at 140 rpm. After 24 h of incubation, mycelia treated with different concentrations of CFS were obtained by centrifugation at 10,000×*g* for 5 min and washed three times with 0.1 M PBS (pH 7.0). Sterile water was used as a negative control. The mycelia were stained with propidium iodide (PI) in the dark for 15 min. Fluorescent images of the mycelia were observed using a laser confocal microscope (excitation light 545 nm, emission light 590 nm).

2.9. Non-targeted metabolomics analysis

Based on the above findings, we selected the control and the two samples treated with 20% CFS for the following metabolomic and transcriptomic studies. Hangzhou Kaitai Biotechnology Co Ltd (China) measured the metabolic bioinformatics data. Briefly, metabolite extraction was performed first for LC-MS detection (Cheng et al., 2023). Liquid chromatography (Thermo, Vanquish) and mass spectrometry (Thermo, Q Exactive Focus) identifications were performed in both positive-ion (ESI+) and negative-ion (ESI-) ion modes. Peak detection, peak filtering, and peak alignment were processed using the R XCMS software package (Navarro-Reig, Jaumot, Garcia-Reiriz, & Tauler, 2015), and the public databases HMDB (Wishart et al., 2022, D622-D631), MassBank (Horai et al., 2010, 703–714), LipidMaps (Sud et al., 2007), Mzcloud (Abdelrazig et al., 2020), KEGG (Ogata et al., 1999), and self-constructed standard libraries were used for substance identification. Quantification of metabolites was obtained by multiple reaction monitoring. Metabolomics data were further analyzed by unsupervised principal component analysis (PCA). Differential metabolites between groups were identified using the projected variable importance (VIP) score of the OPLS model, with the VIP threshold set at 1, $P < 0.05$. These differential metabolites were then mapped to the KEGG pathway database. Each group of samples contained three biological replicates.

2.10. Illumina transcriptome sequencing

Total RNA was extracted using the TR214 Fungal/Bacterial RNA Extraction Kit (Jianshi Biotechnology, Beijing). Library preparation and sequencing were performed by Hangzhou Kaitai Biotechnology Co. (China) on the Illumina Novaseq platform. To more accurately characterize our research subject, we employed the genomic sequence of a specific *A. alternata* strain (Accession number: MT498268.1) as a reference, which is retrievable from the NCBI database at the following link: https://www.ncbi.nlm.nih.gov/nucleotide/MT498268.1?report=genbank&log$=nucltop&blast_rank=1&RID=XZN4RA39016. FPKM values were used as gene/transcript expression in the samples. Differential expression at the transcript and gene level was analyzed using EdgeR. FDR correction of P values was performed to obtain Q values and genes with absolute $Log_2FC \leq 1$, and P values ≤ 0.05 and Q (FDR) values ≤ 0.05 were defined as DEGs and analyzed by KEGG enrichment.

2.11. Comprehensive analysis of metabolome and transcriptome

The KEGG pathway rich in metabolites was selected for correlation analysis with corresponding genes. The Pearson correlation coefficient was used to analyze the correlation between transcriptome and metabolomics data. The heat map is used to show the link between genes and metabolites.

2.12. Statistical analysis

All experiments were conducted in three parallel sessions, and data compilation was done using Microsoft Excel 2019 (Microsoft Corporation, Redmond, WA, USA). Statistical analysis was performed by one-way analysis of variance (ANOVA) using SPSS Statistics Version 23 (IBM Corporation, Armonk, NY, USA), and significant differences between means were identified using Duncan's multiple range test at $P < 0.05$. All data visualizations were created with Origin software, Version 2023b (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. Antifungal activity of E. faecium CFS against A. alternata

As shown in Fig. 1A, the MIC and MFC of CFS on *A. alternata* were 20% and 40%, respectively. From Fig. 1B, it can be seen that the presence of CFS significantly inhibited the colony expansion of *A. alternata*, and this inhibition was dose-dependent. As the concentration of CFS increased, the colony diameter decreased, and 40% CFS completely inhibited the growth of mycelium. The average colony growth rate of the control group (CK) was 9.46 mm per day, and the average colony growth rates of 0.5 MIC and MIC were 6.49 mm and 4.78 mm per day,

(A) MIC and MFC of CFS of *Enterococcus faecium* on *Alternaria Alternata*

Strains	MIC	MFC	2MIC
Alternaria Alternata	20 %	40 %	40 %

(B)

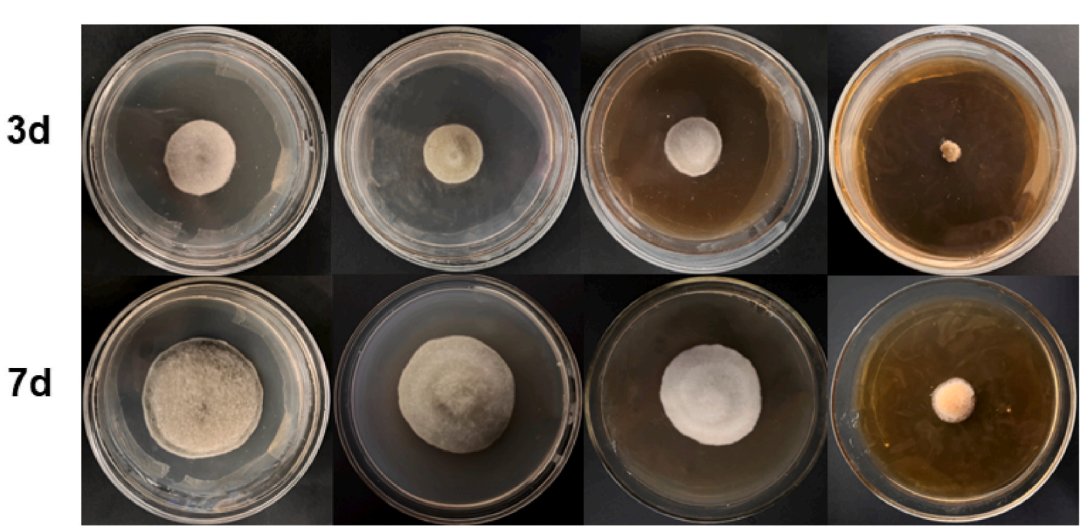

Fig. 1. Fungicidal activity of *E. faecium*. **(A)** MIC and MFC of CFS of *E. faecium* on *A. alternata*. **(B)** Colony size of *A. alternata* after 3 d and 7 d treatment with different concentrations of CFS (0, 0.5, 1, and 2MIC).

respectively. It indicated a clear dose-response relationship between CFS concentration and antifungal efficiency.

3.2. Effect of *E. faecium* CFS on the surface morphology of *A. alternata* mycelium

As shown in Fig. 2A, SEM showed that the control mycelium was morphologically full and structurally smooth and densely organized, *E. faecium* cell-free supernatant-treated fungal hyphae underwent morphological changes, exhibiting significant shrinkage and depression, and when higher concentrations of CFS were applied, the morphological damage was even more severe, with the mycelium being disrupted to the point of fracture under the highest CFS concentration treatment (2 MIC). These preliminary findings may point out that disruption of cell membrane structure may lead to leakage of intracellular macromolecules causing irreversible damage.

3.3. *E. faecium* CFS disrupts the plasma membrane integrity of *A. alternata*

PI is a fluorescent dye that cannot penetrate intact cell membranes but can emit strong fluorescence by binding to nucleic acids through damaged cell membranes. As shown in Fig. 2B, only a minimal amount of weak intermittent red fluorescence was observed in the negative control group, whereas in the CFS-treated group. In contrast, the fluorescence signal increased gradually in a CFS concentration-dependent manner, the red fluorescence was significantly higher and more continuous, and the 2 MIC group possessed the wealthiest and most robust fluorescence, indicating that the cell membranes were damaged after CFS treatment.

By analyzing nucleic acid leakage (indicated by OD260 values), protein leakage (indicated by OD280 values), and relative conductivity, the leakage of proteins (Fig. 2C) and nucleic acids (Fig. 2D) in mycelial suspensions gradually increased with the increase of tetramycin concentration. In addition, as shown in Fig. 2E, the conductivity of the control remained relatively stable, whereas it showed a linear increase with increasing treatment time in the CFS-treated group. After 72 h of treatment, the extracellular conductivity was 21.43% and 36.90% higher than that of the control in the MIC- and 2MIC-treated groups, respectively ($P < 0.05$). This indicated leakage of intracellular electrolytes into the extracellular solution. The relative conductivity of the mycelial suspension increased significantly over time. These results suggest that CFS severely disrupted the cell membrane integrity of *A. alternata*.

3.4. *E. faecium* CFS affects *A. alternata* metabolic profile

In order to better explore the effects of *E. faecium* CFS on the metabolic dynamics of *A. alternata*, we employed UPLC-MS-based metabolic profiling to monitor the changes in metabolites (both primary and secondary). Metabolomics results showed distinct metabolic profiles between the CFS-treated and control groups, with TIC chromatograms (Fig. 3A) highlighting the differences. To determine the differential characteristics of metabolic profiles between the control and CFS treatments, further analyses were performed to screen for differential metabolites. Screening with the p-value and VIP thresholds preset for statistical tests in the results of the primary difference analysis (Kieffer et al., 2016) yielded 243 secondary difference metabolites (Table S1). A volcano plot of the metabolites was drawn by combining the p-value and FC value of each metabolite (Fig. 3B). The results showed that the control (CK) and CFS-treated groups (CFS) metabolites differed significantly. There were 80 metabolites with $P < 0.05$ and VIP >1.0, accounting for 32.92% of the total metabolites. They were classified into 25 categories according to the HMDB database (Table S2), primarily including fatty acyls (14), carboxylic acids and derivatives (13), steroids and their derivatives (6), benzene and substituted derivatives (6), prenol lipids (4), flavonoids (3), organic oxides (3), phenols (3), and organonitrogen compounds (3).

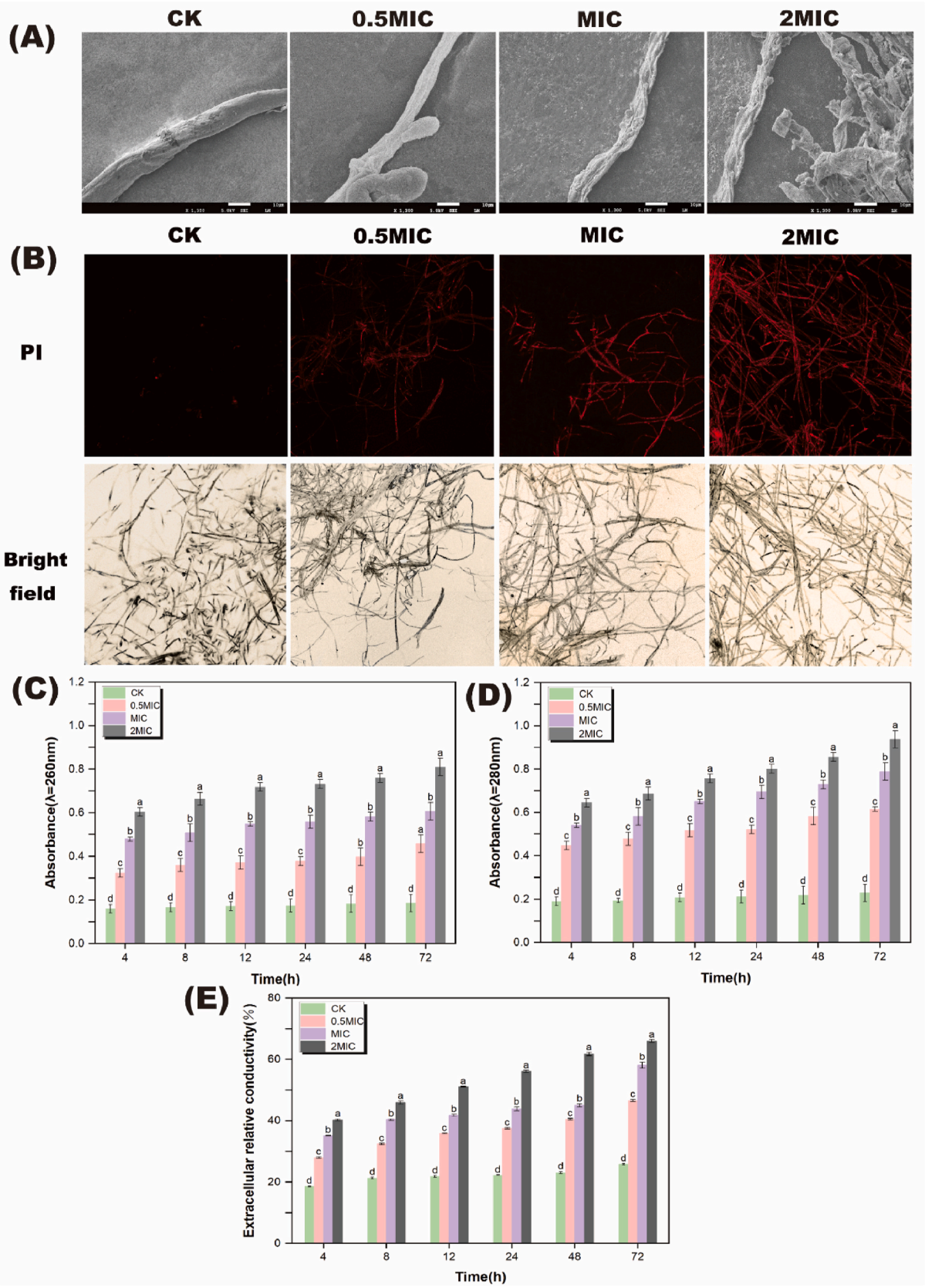

Fig. 2. Physiological changes of *A. alternata* before and after treatment with *E. faecium* CFS. **(A)** Examination of fungal mycelial morphology after 12 h of treatment with different concentrations of CFS, "CK" indicates negative control. **(B)** Mycelium in a bright field and under PI after *A. alternata* was treated with different concentrations of CFS for 24h and stained by PI. Effects of different concentrations of CFS treatments on cytoplasmic leakage of *A. alternata*, including protein **(C)**, nucleic acid **(D)**, and extracellular conductivity **(E)**, according to Duncan's multiple range test ($P < 0.05$), while values carrying different letters indicate statistically significant differences.

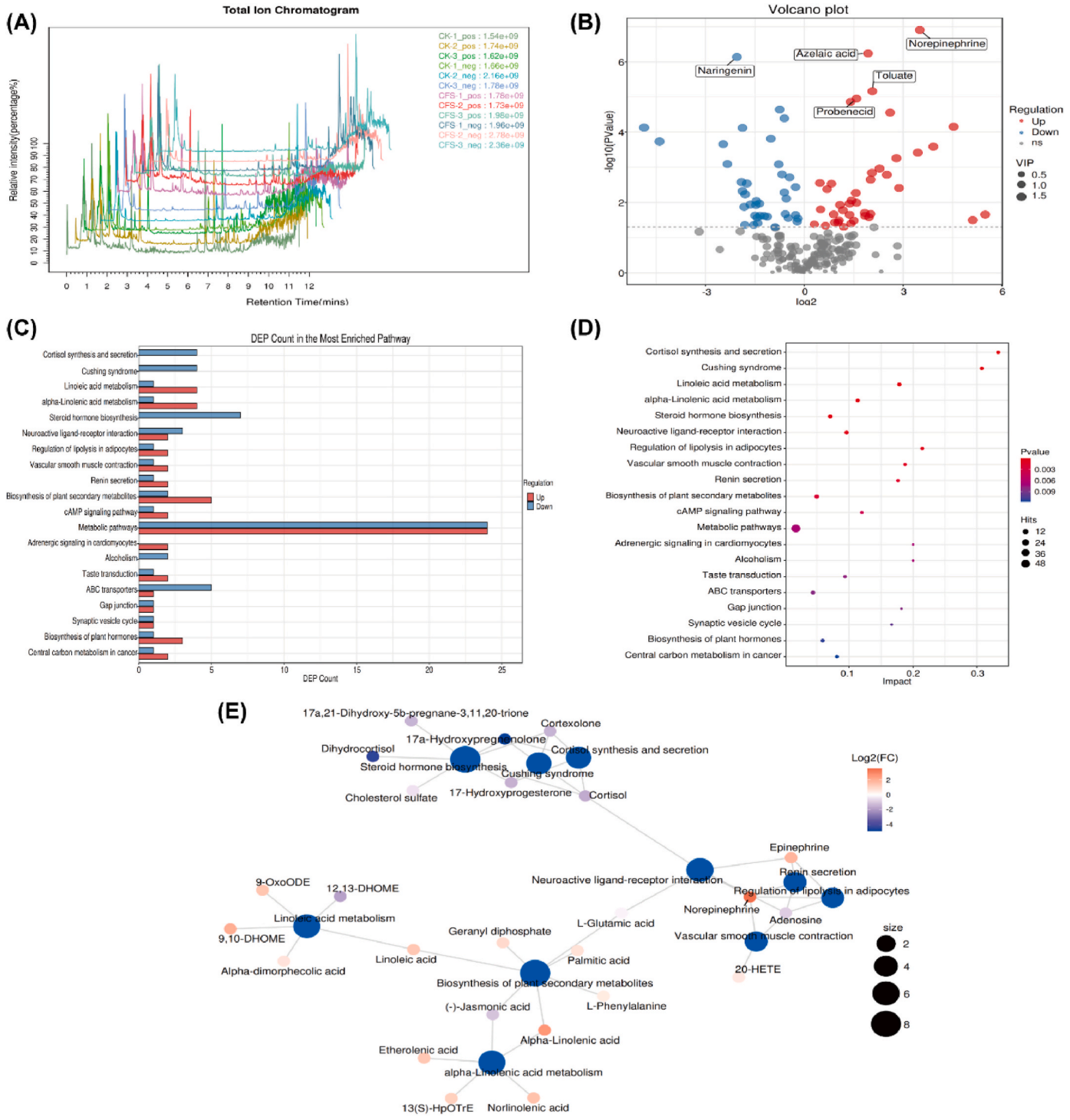

Fig. 3. Changes in metabolic profiles of *E. faecium* CFS before and after its action on *A. alternata* studied by untargeted metabolome sequencing. **(A)** TIC Chromatogram. **(B)** Differential metabolite volcano map. Each point in the image represents a metabolite. Red points represent upregulated differences, blue points represent downregulated differences, and grey points represent metabolites that do not meet the criteria for differential screening **(C)** Statistical map of the number of differentially enriched metabolic pathways. The X-axis represents the number of differential metabolites, and the Y-axis represents different metabolic pathways. Red indicates the number of upregulated, and blue indicates the number of downregulated. **(D)** Metabolic Pathway Influence Factor Bubble Chart. The horizontal axis represents the Impact values enriched in different metabolic pathways, and the vertical axis represents the enriched pathways. The size of the dots indicates the number of metabolites in the pathway. The color is related to the P-value; the redder the color, the smaller the P-value, and the bluer the color, the larger the P-value. **(E)** Network diagram. Blue dots represent pathways, while other dots represent metabolites. The size of the pathway dots indicates the number of connected metabolites; the more connections, the larger the dot. The color of the metabolite dots represents the magnitude of the log2(FC) value, with red indicating upregulation and blue indicating downregulation. The deeper the color, the greater the degree of difference. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Metabolic pathway enrichment analysis of the 243 differential metabolites yielded one hundred and forty-seven metabolic pathways. Among these, the twenty pathways that exhibited the most significant changes were selected for further analysis. As shown in Fig. 3C, most of the differences observed between CFS-treated *A. alternata* and control groups were in energy metabolism, particularly affecting five key metabolic pathways: cortisol synthesis and secretion, Cushing's syndrome-related metabolism, linoleic acid metabolism, steroid hormone biosynthesis, and alpha-linolenic acid metabolism. In Fig. 3D, it is clear that the upregulated metabolites are mainly related to carboxylic acids and their derivatives, including fatty acyls, for example, increased levels of linoleic acid and alpha-linolenic acid are observed. In contrast, downregulated metabolites are predominantly associated with steroids and their derivatives. Of the downregulated metabolites, 17α-hydroxypregnenolone shows the smallest fold change (FC), registering at just 0.03, while levels of both cortisol and dihydrocortisol are significantly reduced. These findings indicate that CFS treatment induces changes in the energy metabolism pathways of *A. alternata*. Using FC and p-values, cytokine localization software was utilized to create an integrated and comprehensive visual representation of the metabolic network, as depicted in Fig. 3E.

3.5. Transcriptome analysis of *E. faecium* CFS acting on *A. alternata*

The molecular changes of *A. alternata* after CFS treatment were investigated by transcriptome analysis; after filtering the raw reads, 52.84 Gb of clean data were obtained for six cDNA libraries (Table S3). The total mapping ratio and uniquely mapping ratio of each library were greater than 79.96% and 80.09%, respectively, indicating that the sequencing quality was high and suitable for further bioinformatic analysis. The gene expression levels of each replicate were evaluated using Principal Component Analysis (PCA) (Fig. 4A), which showed that PCA was reproducible and the intra-group differences were less than inter-group differences, which did not affect subsequent differential gene analysis. Genes with absolute values $Log_2FC \geq 1$ and FDA ≤ 1 were subsequently selected as differentially expressed genes for further analysis (Table S4). The distribution of DEGs was visualized with a volcano plot (Fig. 4B). Compared with the control group, we identified 1295 DEGs in the CFS-treated group, of which 571 genes were up-regulated, and 724 genes were down-regulated. The KEGG pathway annotation results identified 746 differentially expressed genes in fungal metabolism (Fig. 4C), accounting for 57.6%. There were 74, 70, and 61 differentially expressed genes related to Carbohydrate metabolism, Amino acid metabolism, signaling, and cellular processes, respectively. These differentially expressed genes may cause cell metabolism changes

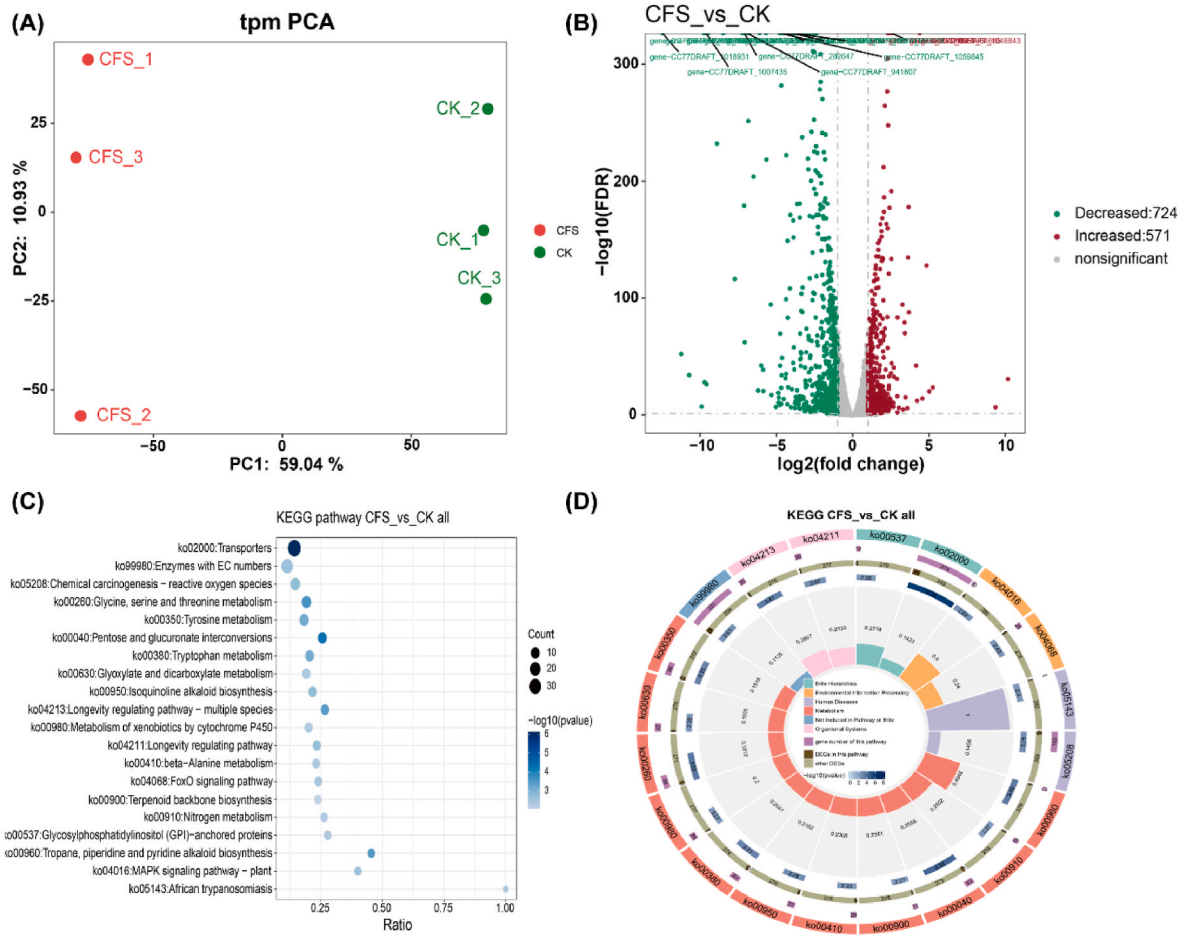

Fig. 4. Transcriptome analysis. (A) PCA score chart. PCA1 represents the principal component with the first highest contribution, and PCA2 represents the principal component with the second highest contribution. The values in parentheses are the contributions of the corresponding principal components. (B) Differential gene volcano map. Each dot in the diagram represents a gene, red indicates increased differential expression, green indicates decreased differential expression, and genes without significant differential expression are represented by grey dots. (C) KEGG enrichment analysis bubble diagram. (D) KEGG enrichment analysis circle diagram. The first circle represents the 20 KEGG IDs with the smallest p-values, the second circle shows the number of genes annotated to that pathway, the third circle displays the differentially expressed genes annotated to that pathway along with other differentially expressed genes, the fourth circle represents the enrichment p-values as -log10§, and the fifth circle indicates the RichFactor. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

by altering gene expression, which affected the growth process of *A. alternata*. Subsequently, KEGG pathway analysis on the DEGs was performed to determine the main metabolic pathways involved. The results showed that all DEGs were classified into 251 KEGG pathways (Table S5), and Fig. 4C showed the top 20 enrichment pathways. In order to facilitate the observation of the distribution of differential genes, the KEGG enrichment analysis circle diagram was used further to display the KEGG result information (Fig. 4D). The results showed that CFS treatment mainly affected the membrane transport function. In this process, 39 related genes changed, followed by the activity of Enzymes with EC numbers, a total of 16 related genes changed, and then Glycine. There were 12 genes changed in the metabolic process of amino acids, such as serine and threonine metabolism. Overall, CFS treatment changed the process of energy metabolism by altering gene expression, which affected the growth process of *A. alternata*.

3.6. Integrated metabolome and transcriptome analysis

Differential characterization of samples at the levels of gene expression level and metabolites is obtained from transcriptomic and metabolomic data. However, transcription and metabolism do not occur independently in biological systems. Previous studies concluded that genes or metabolites involved in the same biological process have the same or similar patterns of change (Zhou et al., 2019). To reveal the mechanisms of regulation and influence between gene expression and metabolites, we analyzed their relationship. Joint KEGG pathway analysis of Metabolites and mRNAs was performed to identify further the metabolic pathways associated with the inhibition of *A. alternata* activity. The results showed that significantly enriched pathways ($p < 0.01$) included the FoxO signaling pathway, Tyrosine metabolism, Steroid hormone biosynthesis, Tryptophan metabolism, etc. (Fig. 5A, Table 1). In order to further link the metabolite profile with the gene expression pattern and to understand the strengths of the positive and negative relationship between the two omics covariances, the metabolites of the above four pathways were selected for correlation analysis with DEGs. The results showed that under the treatment of *E. faecium* CFS, 29 genes in *A. alternata* had transcriptional changes, and 16 metabolites had metabolic changes. We next calculated the Pearson correlation coefficient between gene expression and metabolite abundance. As shown in Fig. 5B, except that the metabolites Norepinephrine and Epinephrine in the Tyrosine metabolism pathway were negatively correlated ($P < 0.05$) with genes Gene-CC77DRAFT_950113, Gene-CC77DRAFT_1032694, Gene-CC77DRAFT_1013212, Gene-CC77DRAFT_1023836, Gene-CC77DRAFT_1097549, all the remaining metabolites and genes were positively correlated. The joint analysis revealed a high correlation between differentially expressed genes and changes in metabolite level.

4. Discussion

Fungal infections represented by *A. alternata* have been one of the critical factors contributing to postharvest losses in the fruit and vegetable industry. Several effective alternative strategies for postharvest disease management have been investigated both domestically and internationally, such as biological fungicides (Wang, Saito, Michailides, & Xiao, 2021) like Natamycin, GRAS compounds (Yang et al., 2017) (which are usually considered safe) and plant-derived fungicides, like plant essential oils. Meanwhile, lactic acid bacteria (LAB) and their metabolites have attracted extensive attention from researchers (Kuley, Metanet, Mustafa, & Yilmaz, 2021; Volentini et al., 2023). LAB has been designated as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration, making LAB a good alternative for replacing synthetic preservatives in foods while minimizing regulatory hurdles and providing consumers with clean-labeled products (Yi et al., 2022). *E. faecium* strains have been used as probiotics and are typically consumed in the form of pharmaceutical preparations. Although reports of *E. faecium* strains having fungistatic effects against fruit and vegetable spoilage fungi are minimal, our current study demonstrated significant antifungal activity of *E. faecium* CFS against *A. alternata in vitro*, with the MIC of *E. faecium* CFS against *A. alternata* being a MIC of 20% in a

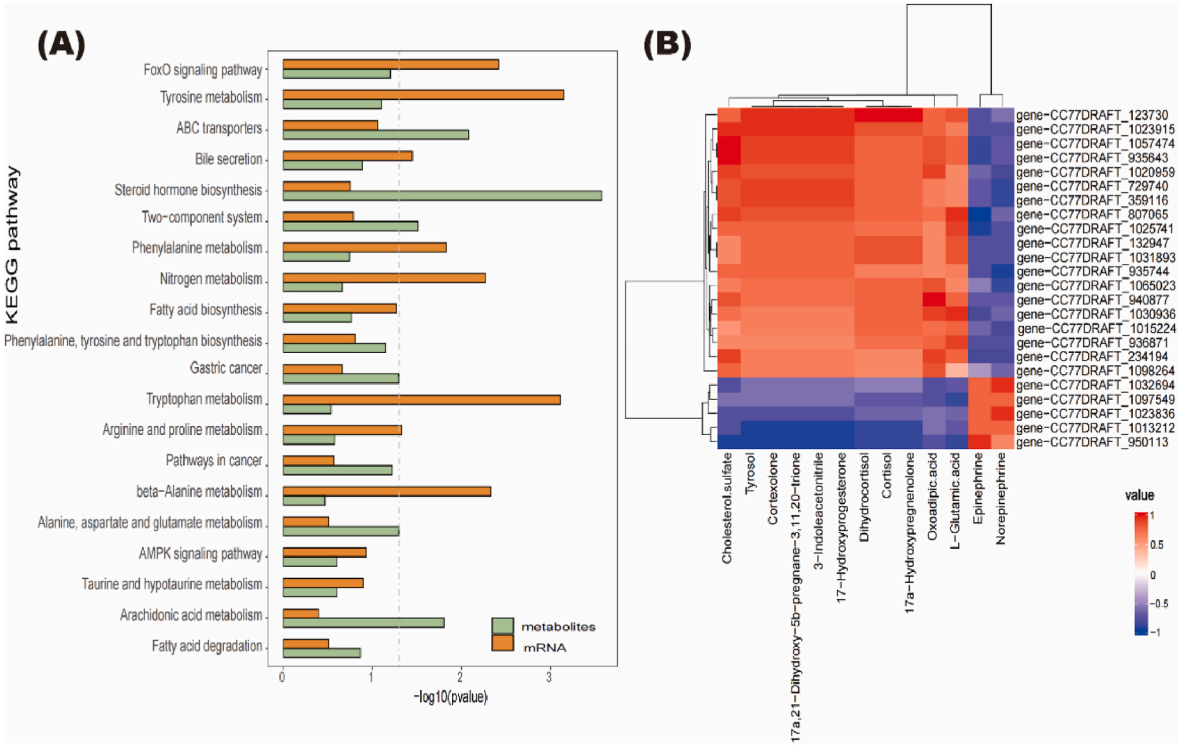

Fig. 5. Integrated metabolome and transcriptome analysis. (A) KEGG pathway enrichment histogram (B) Correlation heat map between gene expression and metabolite abundance of four pathways.

Table 1
Representative DEGs and functions in the comparison of *E. faecium* CFS-treated group and control group.

KEGG pathways	DEM name	Log_2FC	Gene category	Annotated function gene	Log_2FC
Steroid hormone biosynthesis	Cortexolone	−1.47	Gene-CC77DRAFT_950113	S-adenosyl-L-methionine-dependent methyltransferase	1.28
	17-Hydroxyprogesterone	−1.54			
	17a-Hydroxypregnenolone	−4.87			
	Cortisol	−1.64			
	Dihydrocortisol	−4.4			
	Cholesterol sulfate	−0.49			
	17a,21-Dihydroxy-5b-pregnane-3,11,20-trione	−1.46			
Foxo signaling pathway	L-Glutamic acid	−0.20	Gene-CC77DRAFT_1025741	Superoxide dismutase mitochondrial precursor	−2.27
			Gene-CC77DRAFT_1013212	Catalase-domain-containing protein	1.28
			Gene-CC77DRAFT_1023836	Heme-dependent catalase	2.00
			Gene-CC77DRAFT_1097549	Catalase-domain-containing protein	1.27
			Gene-CC77DRAFT_940877	Ras guanine-nucleotide exchange protein Cdc25p	−1.10
			Gene-CC77DRAFT_1015224	Heme-dependent catalase	−1.24
Tyrosine metabolism	Tyrosol	−1.50	Gene-CC77DRAFT_234194	3-dehydroshikimate dehydratase	−6.50
	Norepinephrine	3.50	Gene-CC77DRAFT_936871	Groes-like protein	−1.64
	Epinephrine	1.84	Gene-CC77DRAFT_132947	Copper amine oxidase-like protein	−2.39
			Gene-CC77DRAFT_1057474	Tyrosinase	−1.55
			Gene-CC77DRAFT_1030936	Hypothetical protein CC77DRAFT_1030936	−1.26
			Gene-CC77DRAFT_359116	Hypothetical protein CC77DRAFT_359116	−1.53
			Gene-CC77DRAFT_935643	Di-copper centre-containing protein	−1.19
			Gene-CC77DRAFT_950113	S-adenosyl-L-methionine-dependent methyltransferase	1.28
			Gene-CC77DRAFT_935744	Di-copper centre-containing protein	−1.06
			Gene-CC77DRAFT_1031893	PLP-dependent transferase	−1.59
			Gene-CC77DRAFT_1032694	Di-copper centre-containing protein	1.10
			Gene-CC77DRAFT_1098264	Hypothetical protein CC77DRAFT_1098264	−1.28
Tryptophan metabolism	Oxoadipic acid	−0.61	Gene-CC77DRAFT_1020959	Bifunctional P-450/NADPH-P450 reductase	−1.79
	3-Indoleacetonitrile	−1.83	Gene-CC77DRAFT_1065023	Kynureninase	−3.36
			Gene-CC77DRAFT_1013212	Catalase-domain-containing protein	1.283
			Gene-CC77DRAFT_123730	Indoleamine 2,3-dioxygenase alpha type	−1.04
			Gene-CC77DRAFT_807065	Acetyl-coa acetyltransferase IB	−1.07
			Gene-CC77DRAFT_1023915	Indoleamine 2,3-dioxygenase beta type	−3.12
			Gene-CC77DRAFT_1023836	Heme-dependent catalase	2.00
			Gene-CC77DRAFT_729740	Amidase signature enzyme	−2.80
			Gene-CC77DRAFT_1097549	Catalase-domain-containing protein	1.27
			Gene-CC77DRAFT_1015224	Heme-dependent catalase	−1.24

dose-dependent manner.

PI staining showed that *E. faecium* CFS could disrupt the integrity of the plasma membrane of *A. alternata* mycelium. This was confirmed by the leakage of nucleic acids and proteins as shown by OD changes at 260 nm and 280 nm. In addition, SEM showed that *E. faecium* CFS causes significant shrinkage and indentation on the surface of *A.* alternata fungal mycelium and can even disrupt it to the point of fracture, which may indicate that *E. faecium* CFS causes cytoplasmic loss *A. alternata*. These data suggest that *E. faecium* CFS causes irreversible damage to the *A. alternata* mycelial cell membranes.

To better understand the antifungal mechanism of *E. faecium* CFS against *A. alternata*, we used transcriptomics and metabolomics for our analysis. For the transcriptome, we chose to select six pathways for analysis in which *E. faecium* CFS inhibits the most significant enrichment of *A. alternata* differential genes, including Transporters (ko02000), Pentose and glucuronate interconversions (ko00040), Tyrosine metabolism (ko00350), Tryptophan metabolism (ko00380), beta-Alanine metabolism (ko00410) and FoxO signaling pathway (ko04068). *E. faecium* CFS was found to reduce the gene expression of several *A. alternata* enzymes essential for normal growth, such as superoxide dismutase (SOD), several catalases (CAT), acetyl-CoA, acetyltransferase IB, indoleamine 2,3-dioxygenase (IDO), etc. Among them, a decrease in acetyl-CoA directly affects TCA cycling and oxidative phosphorylation metabolism in *A. alternata* cells and reduces ATP production (Akram, 2014); a decrease in CAT reduces the decomposition of hydrogen peroxide in the cells, which may lead to the production of highly toxic hydroxyl radicals. Highly toxic hydroxyl radicals impair the antioxidant function in *A. alternata* cells; IDO is a crucial immunomodulatory enzyme involved in tryptophan metabolism; the down-regulation of the IDO gene will affect Tryptophan metabolism, which indirectly leads to a

decrease in the production of Tryptophan metabolites-Oxoadipic acid and 3-Indoleacetonitril.

In addition, *E. faecium* CFS also led to a reduction in the expression of several *A. alternata* essential protein genes, such as the expression of the Groes-like protein was downregulated by 1.64 GroEL can utilize the energy of ATP to change its conformation to actively regulate the structure of misfolded intermediates (Zhang et al., 2013), which plays a vital role in protein folding and symbiotic microbe-host interactions and pathogenicity (Kupper, Gupta, Feldhaar, & Gross, 2014), down-regulation of this gene may lead to misfolding of the protein passages and aggregation of exposed hydrophobic surfaces, which can develop from a small number of molecules forming a reversible aggregation, develops into irreversible aggregation, and the occurrence of irreversible aggregation of proteins not only affects the structure and function of cells but even causes cell death (Sigler et al., 1998). Similarly, Copper amine oxidase-like protein, whose expressed gene is down-regulated by −2.39, may lead to slowing down or stopping asymmetric cell division (Jiskrová, Kubalová, & Ikeda, 2015). Differential metabolite changes between CFS-treated *A. alternata* and controls mainly occurred in energy metabolism, the up-regulated metabolites were mainly related to Carboxylic acids and derivatives and Fatty Acyls, and down-regulated metabolites were mainly related to Steroids and steroid derivatives Steroids and their derivatives, CFS treatment resulted in fungal Changes in energy metabolism-related pathways. The metabolomics results showed that the treatment of CFS could change energy metabolism-related pathways in *A. alternata* cells.

To further link the metabolite profiles with gene expression patterns, we selected metabolites from four pathways, FoxO signaling pathway, Tyrosine metabolism, Steroid hormone biosynthesis, Tryptophan metabolism, and DEG for the correlation analysis (Fig. 5, Table 1). In the FoxO signaling pathway, the downstream target genes of the FoxO signaling pathway include SOD, DNA damage repair enzymes, apoptosis-related genes, and others, and the regulation of the expression of these genes is involved in various critical biological functions. For example, in metabolic regulation, activation of the FoxO signaling pathway inhibits the expression of the mitochondrial respiratory chain complex and oxidative phosphorylation, which reduces the cell's ATP content and metabolism level. The FoxO signaling pathway is an essential biological regulatory network that participates in regulating several biological processes, such as metabolic regulation and lifespan regulation (Joob, Yasri, & Wiwanitkit, 2021). Down-regulation of superoxide dismutase mitochondrial precursor expression induces changes in differential metabolite levels in the FoxO signaling pathway and reduces fatty acid oxidation and glycolysis in *A. alternata* cells. In the Tyrosine metabolism pathway, genes encoding phenolic synthesis are significantly down-regulated, leading to down-regulation of the metabolites Tyrosol, Epinephrine, and Norepinephrine; tryptophan is a precursor of many secondary metabolites in microorganisms (Le Floc'H, Otten, & Merlot, 2011), and indole is a critical secondary compound produced from tryptophan. Transcriptomics and metabolomics data show that in the Tryptophan metabolism pathway, 3-Indoleacetonitrile levels are strongly correlated with the expression levels of genes encoding the indoleamine 2,3-dioxygenase alpha type. That indole is a significant contributor to the production of melanin (Henson, Butler, & Day, 1999) (which is usually composed of polymerized phenolic and/or indole compounds); melanin is vital in microbial pathogenesis because it is involved in appressorium formation, host cell production of scavenging ROS to protect the fungus from environmental stresses, and induction of host immune responses. In the Tyrosine metabolism pathway, genes encoding phenolic synthesis are significantly down-regulated, leading to down-regulation of the metabolites Tyrosol, Epinephrine, and Norepinephrine; tryptophan is a precursor of many secondary metabolites in microorganisms (Le Floc'H, Otten, & Merlot, 2011), and indole is a critical secondary compound produced from tryptophan. Transcriptomics and metabolomics data show that in the Tryptophan metabolism pathway, 3-Indoleacetonitrile levels are strongly correlated with the expression levels of genes encoding the indoleamine 2,3-dioxygenase alpha type. That indole is a significant contributor to the production of melanin (which is usually composed of polymerized phenolic and/or indole compounds); Melanin is important in microbial pathogenesis because it is involved in attachment formation, scavenging of ROS produced by host cells to protect the fungus, protection against environmental stresses, and induction of host immune responses (Ma et al., 2017). The reduction of indole content in *A. alternata* cells after CFS treatment may directly inhibit melanin (Zhang et al., 2022a). In the Steroid hormone biosynthesis pathway, the reduction of 17-Hydroxyprogesterone, 17a-Hydroxypregnenolone, and the gene controlling the expression of 3-dehydroshikimate dehydratase may inhibit the expression of progesterone (also known as progesterone) production, a common precursor to many hormones, reduces hormone biosynthesis in *A. alternata* cells, leading to growth inhibition in *A. alternata*. Significantly lower levels of steroid hormones reduce binding to the nuclear receptor (NC) in *A. alternata* cells, which reduces the regulation of gene expression in *A. alternata*.

5. Conclusion

E. faecium CFS showed good antifungal activity against *A. alternata*. Specifically, *E. faecium* CFS disrupted the cell wall and cell membrane integrity of *A. alternata*, resulting in cell membrane distortion, rupture, and leakage of cell components in mycelia. In addition, the transcriptome and metabolic analyses showed that *E. faecium* CFS treatment changed the energy metabolism process of *A. alternata*. The comprehensive analysis of two omics data revealed that *E. faecium* CFS could also inhibit the growth of *A. alternata* by reducing ATP production in cells, damaging cellular antioxidant function, reducing biosynthesis of hormones in cells, and decelerating or arresting the division of asymmetrical cells. Although the intracellular metabolic mechanism of fungi are complex and changeable, our current data can substantiate that *E. faecium* CFS has an inhibitory effect on fruit and vegetable spoilage induced by *A. alternata*, which could be leveraged to develop an eco-friendly preservative for these foods, thus reducing the on chemical preservatives and enhancing food quality.

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CRediT authorship contribution statement

Lu Wang: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Shasha Jiang:** Formal analysis, Methodology, Resources, Supervision, Writing – review & editing. **Dehai Li:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. **Changyan Sun:** Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.103442.

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ARTICLES FOR FACULTY MEMBERS

EFFECT OF LACTIC ACID BACTERIA AS BIO-PRESERVATION AGAINST SPOILAGE FUNGI FROM PAPAYA FRUIT

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Effect of lactic acid bacteria and *Bacillus* on anthracnose disease in postharvest papaya fruit

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Abstract. *Thi QVC, Dung TQ, Huynh NTN, Truc NT, Thuy NP. 2023. Effect of lactic acid bacteria and* Bacillus *on anthracnose disease in postharvest papaya fruit. Biodiversitas 24: 5883-5894.* Anthracnose disease of papaya fruit caused by *Colletotrichum* (isolate TD1), identified by 5.8S rRNA sequence homology, is a main obstacle in papaya fruit, influencing fruit quality and minimizing shelf life. Therefore, to diminish the disease and sustain the fruit quality, the impact of Lactic Acid Bacteria (LAB) and *Bacillus* on the growth of anthracnose disease and fruit quality was investigated *in vitro* and *in vivo*. Furthermore, 13 LAB and 12 *Bacillus* isolates were collected from traditional fermented vegetables and papaya rhizospheric soils. The results showed that 13 LAB isolates and 6 isolates of *Bacillus* were inhibitory against *Colletotrichum* in an *in vitro* test. Two isolates of LDC11 and BHL21 with the highest antifungal activity were selected to evaluate their effect on *Colletotrichum* growth and papaya fruit quality under *in vivo* conditions. These findings indicated that the isolates LDC11 and BHL21 at a density of 10^6 CFU/mL reduced the disease incidence and severity. The LAB and *Bacillus* treated papaya fruit increased a number of parameters, such as weight loss, TSS, and L*, a*, and b* values. However, vitamin C content, TA, and fruit firmness were reduced compared to the control. The research shows a potential applications of LAB and *Bacillus* in the postharvest preservation of papaya fruit. To our knowledge, this is the first study to apply *Bacillus* and LAB bacteria to control diseases in postharvest papaya in Vietnam.

Keywords: Anthracnose, *Colletotrichum*, lactic acid bacteria, postharvest disease

INTRODUCTION

Papaya (*Carica papaya* L.) is a crucial fruit in many tropical and subtropical countries worldwide. Papaya fruit contains vitamins A, E, and C and antioxidant compounds (Alara et al. 2022). However, this fruit is susceptible to fungal diseases, especially anthracnose caused by *Colletotrichum* spp. during the postharvest stage (Gabrekiristos and Dagnew 2020). The fungus that attacks fruit usually forms brown spots on the peel, penetrating the fruit flesh and causing it to rot, become perishable, and suffer damage during storage (Saini et al. 2017). According to Hodges et al. (2011), microbial attack is responsible for approximately 30% of postharvest losses of fruits and vegetables in developing nations. Significant losses can be attributable to fungal invasions during storage and transit (Liu et al. 2013).

Until now, farmers mainly used fungicides to prevent and treat fungal diseases on fruit, including anthracnose caused by *Colletotrichum* spp. in pre- and postharvest papaya fruit (Qadri et al. 2020). The abuse of chemical drugs pollutes the environment, affects consumers' health, and reduces the value of exported agricultural products (Igbedioh et al. 1991). Heat treatment and antifungal drugs are the two most widely used ways to prevent postharvest fungal diseases (Feliziani and Romanazzi 2013). The quality of preserved fruit is not uniform and is frequently harmed by heat because managing heat dispersion during processing is a challenging and unresolved problem for heat treatment methods. On the contrary, using antifungal drugs poses health risks to consumers, environmental pollution, and many pathogens develop pesticide resistance (Vitiello et al. 2023). Furthermore, natural foods devoid of chemical residues are also demanded by consumers (Valencia-Chamorro et al. 2011). As a result, numerous studies have been conducted to look for alternative preservation solutions to minimize harmful effects during storage and preserve food quality (Saucedo-Pompa et al. 2007; Saucedo-Pompa et al. 2009).

In recent years, the trend of postharvest preservation of agricultural products by biological means, such as the use of microorganisms and/or their metabolic products to avoid spoilage and lengthen the shelf food life, has attracted the attention of many scientists (Zubrod et al. 2019). The use of edible coatings, particularly on highly perishable products, is one of the suggested strategies for extending the shelf life of fruit (Yadav et al. 2022). There have also been reports of the biological control of postharvest infections using antagonistic agents, such as bacteria or yeasts, that are inhibit to pathogenic microbes (Liu et al. 2013; Buchholz et al. 2018; Verma et al. 2022). Many previous research results have shown the inhibit influence of LAB and *Bacillus* on many fungal diseases on postharvest fruits (Ghosh et al. 2015; Neelima et al. 2016;

Lastochkina et al. 2019). According to Belkacem-Hanfi et al. (2014), LAB species from stored wheat samples showed high inhibitory effects against *Aspergillus carbonarius*. In a study by Cavaglieri et al. (2005) revealed that ten *Bacillus* strains could inhibit *Fusarium verticillioides* growth, a soil-borne pathogen in maize. The study by De Simone et al. (2021) showed that the cell-free supernatants of two *Lactiplantibacillus plantarum* strains contrasted the growth of *Botrytis cinerea*, responsible for the cause of fruit and vegetable spoilage phenomena in postharvest kiwi fruits. Until now, however, little information has been noticed about the isolation and antifungal activity of LAB and *Bacillus* against *Colletotrichum* spp., which seriously causes anthracnose disease in postharvest fruits in Vietnam. In general, most previous studies have focused on determining the antifungal activity of LAB and *Bacillus* under *in vitro* conditions, while the control of fungal diseases and postharvest quality of papaya fruit have not been studied. Therefore, this goal was to isolate and evaluate the influence of LAB and *Bacillus* on the *Colletotrichum* growth and quality of postharvest papaya fruits.

MATERIALS AND METHODS

Sampling, isolation, and identification of *Colletotrichum* spp.

Colletotrichum spp. was isolated from anthracnose papaya fruit with typical symptoms such as a large, sunken circular wound with a dark brown to black color. The infected papaya fruits were collected from different regions of Vinh Long Province, the Mekong Delta, Vietnam. The fungi were isolated according to the tissue isolation method of Cai et al. (2009). In brief, the diseased papaya fruit was cleaned with a running tap and distilled water many times. Next, the diseased parts were cut into 1 x 1 cm pieces and sterilized with 70% ethanol for 90 sec. The samples were then rinsed three times with sterile distilled water. After that, these segments were placed aseptically in a potato dextrose agar plate (PDA: 200 g/L potato, 20 g/L glucose, and 20 g/L agar, pH = 7.0). Finally, the dishes were incubated at 30ºC for 3-5 days. The fungus *Colletotrichum* was identified based on morphological characteristics (Barnett and Barry 1972), PCR technique, and sequencing of the 5.8S gene fragment with primers ITS1: 5′-TCC GTA GGT GAA CCT GCG G-3′ and ITS4: 5′-TCC TCC GCT TAT TGA TAT GC-3′ (White et al. 1990). PCR products were purified and sequenced by Macrogen Company, Korea (www.macrogen.com).

The isolated fungi were tested the pathogenicity by inoculating experiments on healthy fruits (Jimenez et al. 1993; Marin et al. 1996). In brief, healthy and uninjured fruits were washed with running water many times. The fruits were then sterilized on their surfaces using 70% ethanol and rinsed thrice with sterile distilled water. Next, the fruits were inoculated with the fungus *Collectotrichum* at a 10^5 spore/mL concentration. The inoculated fruits were kept with wet tissue to maintain humidity and incubated in a dark place. Finally, the pathogen was reisolated and identified from the diseased portion to prove Koch's postulates.

Sample collection, isolation, and identification of LAB and *Bacillus*

LAB isolates were isolated from fermented vegetables and purchased at traditional markets in Vinh Long province of the Mekong Delta, Vietnam. LAB was isolated, according to Magnusson et al. (2003). Briefly, 10 mL or 10 g of samples were added to the 90 mL sterile water. Then, the mixture was serially diluted to 10^{-6}. Finally, the sample from each dilution was spread on MRS (De Man et al. 1960) with the following compositions: 20 g/L glucose, 10 g/L peptone, 2 g/L ammonium citrate, 5 g/L yeast extract, 10 g/L meat extract, 5 g/L sodium acetate, 0.05 g/L manganese sulfate tetrahydrate, 5 g/L dipotassium phosphate, and 0.1 g/L magnesium sulfate heptahydrate (pH = 6.5) and incubated at 37ºC for 24-48 hours.

Bacillus bacteria was isolated from papaya rhizospheric soils in Vinh Long province of the Mekong Delta, Vietnam. Bacteria were isolated, according to Ashwini and Srividya (2014). Briefly, 10 g rhizospheric soils were diluted in 90 mL sterile water and heated at 80°C for 20 min. Then, the mixture was serially diluted to 10^{-6}. A sample from each dilution was then streaked on nutrient agar (NA) with the following compositions: 0.5 g/L peptone, 0.5 g/L sodium chloride, and 0.2 g/L yeast extract (pH = 7.0) and incubated at 37°C for 24 h.

LAB and *Bacillus* were identified with morphological and biochemical characteristics such as Gram staining and spore staining, oxidase, and catalase reactions (Harrigan and McCance 1976) and identified by PCR reaction and 16S rRNA gene fragment sequencing with primers 27F: 5'-AGA GTT TGA TCM TGC TCA G-3' and 1492R: 5'-TAC GGY TAC CTT GTT ACG ACT T-3' (Heuer et al. 1997).

Antifungal activity of isolated bacterial isolates against *Colletotrichum*

The mycelium-and-spore antagonistic method was used to test the antifungal activity of LAB and *Bacillus* against the fungus *Colletotrichum*.

Antifungal activity of LAB isolates against *Colletotrichum*

The dual culture method (Lahlali et al. 2020) evaluated mycelial growth inhibition with minor modifications. Briefly, bacteria were inoculated into two 2 cm lines on PDA agar plates. Next, using a cork borer, a mycelial plug (0.5 x 0.5 mm) was cut and placed within bacterial bands in the center of the plate. The inhibitory activity was recorded after five to seven days of incubating at 30°C. Plates with only mycelial plugs were used as a negative control.

The dual culture overlay method was used to detect the spore inhibitory activity of LAB against *Colletotrichum* (Magnusson and Schnürer 2001). LAB isolates were inoculated in two parallel lines (the length of line is 2 cm) on MRS agar plates and anaerobically incubated at 30°C for 24 h. The plates were overlaid with 2 mL of PDA medium (0.7% agar) supplemented with *Colletotrichum*'s spores at 10^6 spores/mL density. The plates were incubated

at 37°C for 48 hours. The inhibition zone diameter (d) was recorded and scaled as follows: d ≤ 2 mm: weak activity; 21 mm ≤ d ≤ 40 mm: average inhibition; 41 mm ≤ d ≤ 60 mm: strong inhibition; and d > 61 mm: very strong activity (Muhialdin et al. 2018).

Antifungal activity of *Bacillus* isolates against *Colletotrichum*.

The mycelium inhibitory activity of *Bacillus* against *Colletotrichum* was tested by the dual culture method described above. In this study, the agar well diffusion method was used to evaluate the spore inhibitory activity of *Bacillus* (Dhanasekaran et al. 2012). In brief, *Bacillus* bacteria were cultured in nutrient broth (NB) medium for 24 h. Then, the culture solution was centrifuged at 10,000 rpm, at 4°C for 10 min, and collected the supernatant. The fungal suspension (100 µL) with *Colletotrichum*'s spores was spread onto the agar surface at 10^6 spores/mL density. Next, a 5 mm cork borer was used to perforate the well on PDA agar plates. Then, the supernatant (80 µL) was added to the wells, and the plate was incubated at 30°C for 24 h.

Effects of LAB and *Bacillus* on the growth of anthracnose and quality of postharvest papaya fruits

The effect of LAB and *Bacillus* on anthracnose disease and postharvest quality of papaya fruits was carried out according to Yadravi et al. (2022) with some modifications. The papaya fruit weighs around 500-800 g and is harvested at the green ripe stage with 1-2 yellow streaks on the surface (8-9 months after fruiting). Fruits were selected with uniform size, shape, and ripeness without any signs of mechanical wounding, insect damage, or disease for this experiment. LAB was cultured in MRS, while *Bacillus* were cultured in NB medium and incubated at 30°C for 24 h. Bacterial density was then determined by spectrophotometric measurements at 600 nm (Kim et al. 2021), where $OD_{600\ nm}$ = 1.0 corresponds to a bacterial density of 10^8 CFU/mL. Finally, based on the survey results, the papaya fruits were immersed in a solution of LAB and *Bacillus* for one minute with the following treatments:

+ Control treatment: papaya fruit was not treated with bacteria.

+ LAB treatment: papaya fruit was treated with isolate LDC11 at a 10^6 CFU/mL density.

+ *Bacillus* treatment: papaya fruit was treated with isolate BHL21 at a 10^6 CFU/mL density.

The treatments were arranged in a completely randomized design (CRD), and each treatment was repeated thrice with 44 fruits/treatment. Papaya fruit is stored at a temperature of 25°C until 12 days. Samples will be collected periodically every 3 days during storage to analyze the following parameters:

Disease incidence (%): the fruits are observed for the disease symptoms, and the number of infected fruits is recorded on days 0, 3, 6, 9, and 12 during the study (McMillan 1986).

The disease severity: the extent of infection is scored using a 0-4 scale (Hofman et al. 1997) as follows: 0 = No infection, 0.1-25.0% fruit surface infected = 1, 25.1-50.0% fruit surface infected=2, 50.1-75.0% fruit surface infected = 3 and 4 = >76.0% fruit surface infected.

Weight loss: a 4-odd electronic is used as balance; papaya fruits are weighed at the start of the experiment (0 days) and every 3 days interval of storage (12 days) (Khaliq et al. 2015).

Total soluble solids (TSS): According to Zhang et al. (2008), TSS is calculated by determining each fruit's refractive index using a handheld refractometer (Atago Master 20T, Japan), and the findings are represented as percentages (%).

Titratable acidity (TA): The total acid content of the juice is measured by titrating with 0.1 N NaOH (Shi et al. 2018).

Vitamin C: According to Roe et al. (1948), the vitamin C content is determined; the results represented in mg of ascorbic acid per 100 g fresh weight.

The fruit firmness: the samples are analyzed using a texture analyzer (Food Technology TMS-Pro, USA). The firmness of the flesh on two opposite sides of each fruit was measured and recorded as N (newtons). For each fruit, the firmness is determined by averaging three readings.

Fruit color: A colorimeter is used, and the peel's color is determined (Hunter Lab, MH-C800 4500L, USA). This is determined at three locations on the fruit: L* (range from 0 to 100 for white) and a* (positive values for red, negative values for green) lightness levels are calculated.

Statistical analysis

The data were processed using Microsoft Excel and Statgraphic 16.1 software. The BLASTn tool was used to compare the similarity of LAB and *Bacillus* with fungal strains in the NCBI database. A neighbor-joining phylogenetic tree was constructed using the MEGA 5.1 software and the Kimura-2 model with 1,000 replications. One-way analysis of variance (ANOVA) and Tukey's multiple range tests were used to examine the treatment differences with a 95% confidence level.

RESULTS AND DISCUSSION

Isolation and identification of *Colletotrichum*

In this study, two isolates of *Colletotrichum* (namely, isolates TD1 and TD2) were isolated on a PDA medium from anthracnose-diseased papaya fruits (Figure 1.A). The findings revealed that the fungal colonies of two isolated fungal isolates reached 60-70 mm in diameter (Figure 1.B) after 7 days, and the thick mycelium was white. After 10 days of culture, an orange concentric circle was formed inside the mycelium, and oil droplets were mixed in the mycelium (Figure 1.C). Under the microscope, the results showed that the mycelium had no septum, and the spores of the two fungal isolates were cylindrical, one pointed and the other round (Figure 1.D). The mycelium and spore morphological characteristics of the fungus *Colletotrichum* in this study are consistent with the fungus *Colletotrichum* isolated from dragon fruit (Pei et al. 2020) and papaya fruit (Marquez-Zequera et al. 2018).

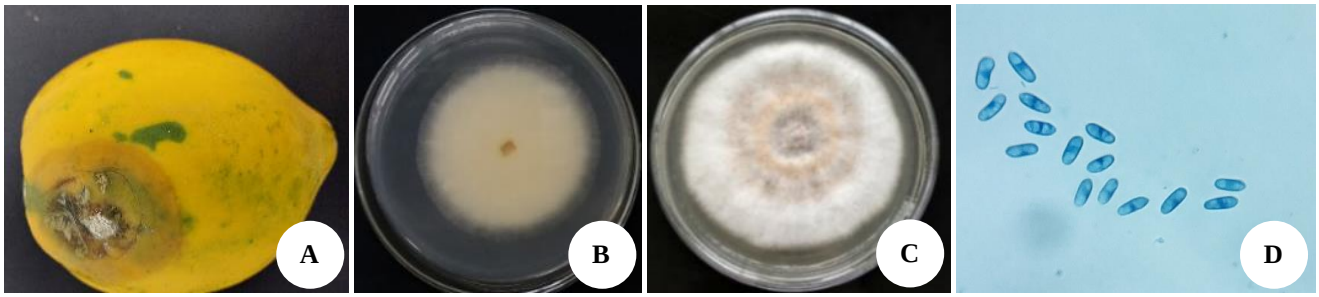

Figure 1. Isolation of the fungus *Colletotrichum.* A. Infected papaya fruit, B-C. Colony of *Colletotrichum*, D. Microscopic appearance

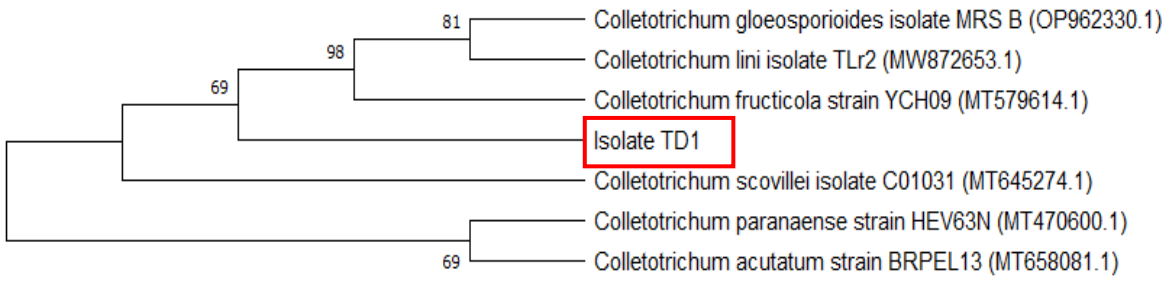

Figure 2. Phylogenetic tree of *Collectotrichum* isolate TD1 based on ITS regions of ribosomal DNA sequences (bootstrap values are given at branching points)

Figure 3. Isolation of LAB from traditional fermented vegetables. Note: A. The LAB's colony isolated on MRS medium, B. The clearance zone appeared around the bacterial colonies, C. Gram stain image of LAB (100X)

The infection showed that two fungal isolates gave positive results, and symptoms appeared on fruits within 3-5 days after inoculation. These features are similar to the symptoms of anthracnose on papaya fruit (Kimaru et al. 2018) and avocado fruit (Aktaruzzaman et al. 2018). Reisolation and identification were made from artificially inoculated papaya fruits that showed typical signs of lesions, and reisolated fungi were similar to those of the original ones. In addition, the infection results revealed that the isolate TD1 has stronger virulence than the isolate TD2. This isolate was selected for sequencing and performing the following experiments. Sequencing results showed that the fungal isolate TD1 was 99.25% homologous to *C. fructicola* YCH09 (MT579614.1) in the NCBI database. The phylogenetic tree showed that isolates TD1 were distributed into separate clusters (Figure 2).

Isolation of LAB

The study isolated thirteen LAB isolates from traditional fermented vegetables. Most colonies were round (Figure 3.A), and the clearance zone appeared around the bacterial colonies when the medium was supplemented with $CaCO_3$ (Figure 3.B). In the study, all isolated bacterial isolates were Gram-positive, rod-shaped (Figure 3.C), non-spore-forming, and had negative catalase and oxidase reactions. The findings indicated that the morphological and biochemical characteristics of isolated LAB are similar to the previous results published by Arimah et al. (2014). A report by Zakaria et al. (2018) showed that 30 strains from fermented catfish had a clear zone around the colony and were gram-positive with bacilli-shaped, catalase-negative, and oxidase-negative.

Antagonistic activity of LAB against *Colletotrichum*

The results demonstrated that 13 LAB isolates inhibited the germination of spores and mycelium elongation of *Colletotrichum*, of which 4 isolates (30.7%) had strong antagonistic activity, 6 isolates (46.1%) had moderate antagonistic activity, and 3 isolates (30.7%) showed weak antifungal activity (Figure 4). The findings also revealed

that isolate LDC11 has the highest antagonistic activity against *Colletotrichum*, with a 62.8% inhibition of fungal growth. Recent studies have shown that LAB inhibits fungi because they produce certain substances that inhibit the growth of spoilage molds on fruits and foods (Lindgren and Dobrogosz 1990). El-Mabrok et al. (2012) reported that 2 species of *L. plantarum* and *L. paracasei* strongly prohibited the germination of *C. gloeosporioides*, which infects chili seeds. The research of Cheong et al. (2014) collected 897 strains of LAB, of which 12 strains (1.3%) indicated inhibitory activity against *Penicillium commune*, the common cheese spoilage mold, inhibiting its growth by more than 60%. Lipińska et al. (2016) investigated the inhibitory activity of several LAB species; they discovered that *L. pentosus* and *L. plantarum* strongly inhibit the mycelial growth of *F. latenicum*, *A. niger*, *Alternaria alternata*, and *A. brassicicola*. Barrios-Roblero et al. (2019) isolated 10 LAB strains from 2 fermented beverage samples (Tepache and Te). By this investigation, all strains prohibited spore germination by at least 60% and mycelium growth by 100%. The recent study by Steglińska et al. (2022) revealed that most LAB isolates inhibit a wide range of antifungal activity spectra, consisting of 10 phytopathogens: *C. coccodes*, *Pectobacterium carotovorum*, *Streptomyces scabiei*, *A. solani*, *A. tenuissima*, *A. alternata*, *Phoma exigua*, *Rhizoctonia solani*, *F. oxysporum*, and *F. sambucinum*.

Sequencing results showed that the isolate LDC1 and LDC11 were 99.69 and 100% similar to *Lactiplantibacillus plantarum* strain KB-25 (MT378128.1) and *Lactiplantibacillus plantarum* strain 1929 (MT597746.1) in the GeneBank. The phylogenetic tree showed that 2 isolates LDC1 and LDC11 were distributed into the same clusters (Figure 5).

Isolation of *Bacillus*

Moreover. 12 *Bacillus* isolates were collected from 3 papaya rhizosphere soil samples. The results showed that the bacterial colonies were usually milky white with wrinkled large, smooth small, and smooth large colonies (Figure 6.A). The findings showed that the isolates were gram-positive, long rod-shaped (Figure 6.B), spore-forming (Figure 6.C), catalase- and oxidase-positive. The current study indicated that the morphological and biochemical characteristics of isolated *Bacillus* agree with the previous results of Ashwini and Srividya (2014). From the rhizosphere of *Coffea arabica* L., the research of Kejela et al. (2016) showed that all isolated *Bacillus* species were gram-positive, catalase-positive, spore-forming, rod-shaped, and able to survive at 80°C.

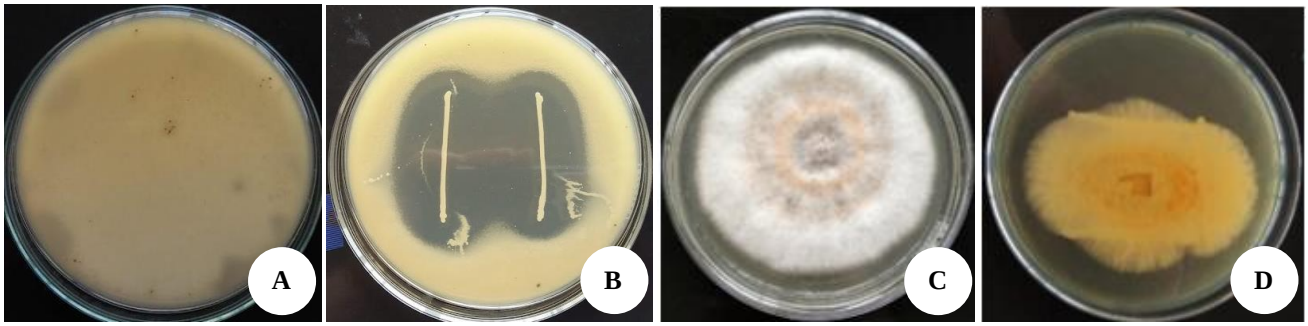

Figure 4. Antagonistic activity of LAB against *Colletotrichum* (isolate TD1). Note: Spore antagonistic activity of LAB against *Colletotrichum* (isolate TD1) at day 3: (a). Control, and (b). Isolate LDC11; mycelial antagonistic activity of LAB against *Colletotrichum* (isolate TD1) at day 3: (c). Control, and (d). Isolate LDC11

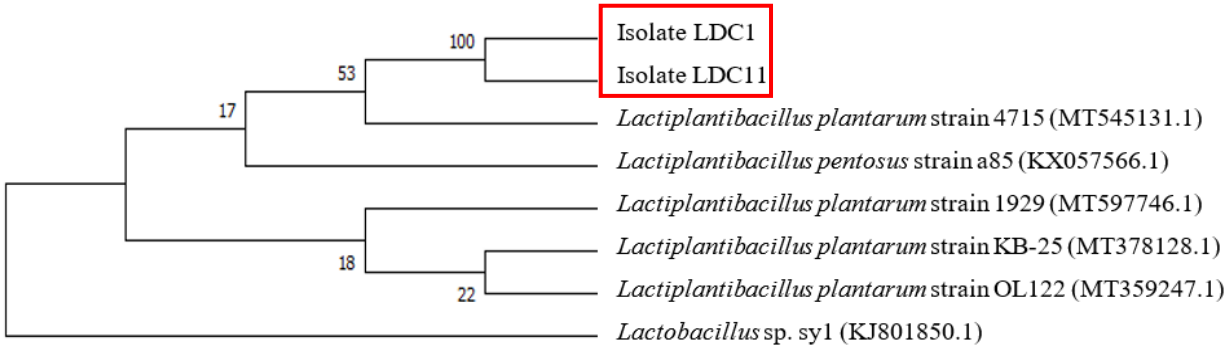

Figure 5. Phylogenetic tree of two isolates LDC1 and LDC11, based on 16S rRNA sequences (bootstrap values are given at branching points)

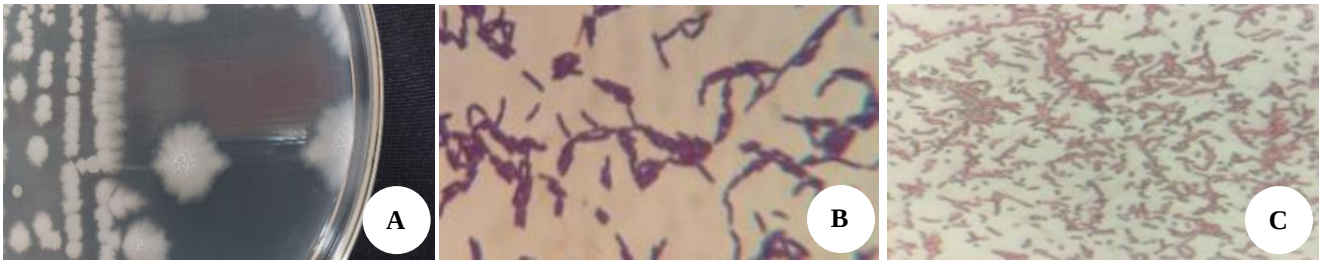

Figure 6. *Bacillus* isolated from papaya rhizospheric soils. Note: A. *Bacillus*' colony on NA medium (isolate BHL21), B. Gram stain image of isolate BHL21 (100X), C. Spore stain image of isolate BHL21 (100x)

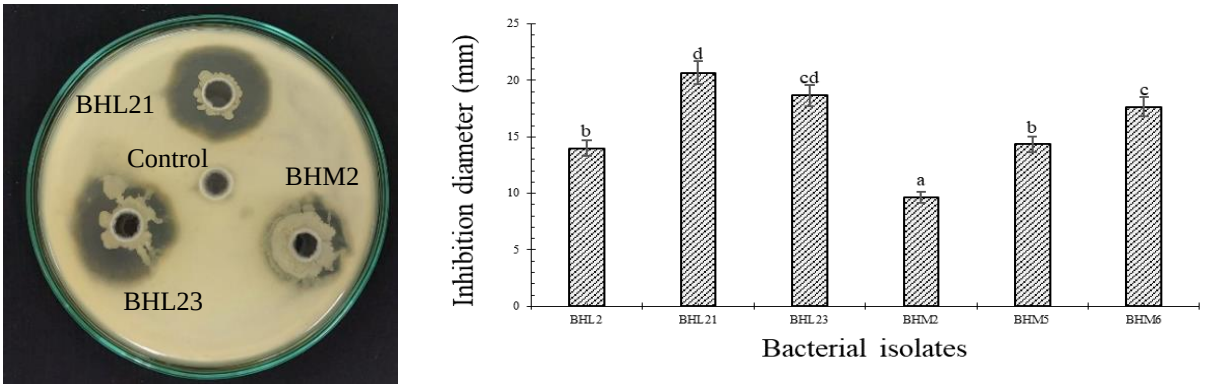

Figure 7. Mycelial inhibitory activity of *Bacillus* against *Colletotrichum* (isolate TD1). Note: Vertical bars represent SE, means followed by different letters are statistically significant ($p<0.05$)

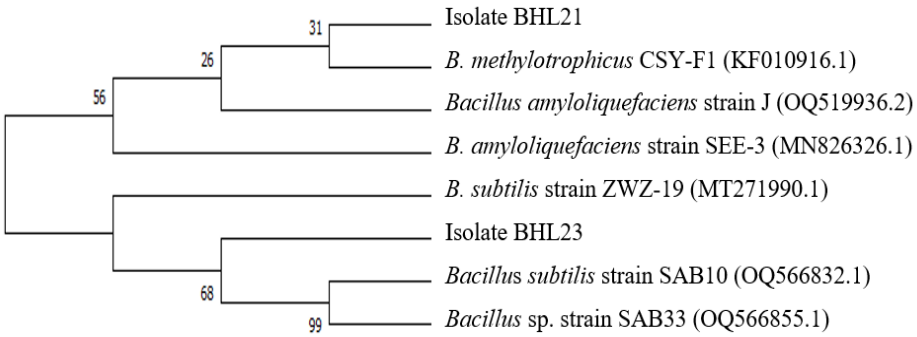

Figure 8. Phylogenetic tree of two isolates BHL21 and BHL23, based on 16S rRNA sequences (bootstrap values are given at branching points)

Antagonistic activity of *Bacillus* against *Colletotrichum* (isolate TD1)

The findings showed that 6/12 *Bacillus* isolates had spore and mycelial antagonistic activity against *Colletotrichum* of which 2 isolates, BHL21 and BHL23, exhibited the highest activity with inhibition diameters of 20 mm and 18 mm, respectively (Figure 7). The results of Hailmi et al. (2017) showed that *Bacillus* sp. (UniSZA-DA) strongly inhibited the growth of *C. gloeosporioides*, which causes anthracnose on papaya grown in Malaysia, by an average of 58.89 ± 2.72% after 7 days of incubation for the control. Research by Gao et al. (2018) showed *B. subtilis* CF-3 exhibited antifungal activity against fungal diseases on peaches and litchi, such as *Botrytis cinerea*, *C. gloeosporioides*, *Penicillium expansum*, *Monilinia fructicola*, and *Alternaria alternata*. Similarly, the results of Girish and Prabhavathi (2019) showed that *B. amyloliquefaciens* (MTCC 10439), *B. cereus* (MTCC 9017) effectively inhibited the growth of the fungi *C. gloeosporioides* and *C. carica papayae and* showed a zone of inhibition or reduction of mycelium growth compared with the control after 10 days of incubation by the dual culture method.

Sequencing results showed that isolates BHL21 and BHL23 were 99.63 and 99.09% homologous to *B. methylotrophicus* CSY-F1 (KF010916.1) and *Bacillus amyloliquefaciens* strain J (OQ519936.2*)* in the NCBI

database. The phylogenetic tree showed that 2 isolates BHL21 and BHL23 were distributed into 2 distinct clusters (Figure 8).

Effect of isolates LDC11 and BHL21 on disease incidence in papaya fruit

The research showed that papaya fruit treated with two isolates of LDC11 and BHL21 reduced the disease incidence and significantly differed from the control (Figure 9.A). By day 3, the two bacterial treatments did not show any infection symptoms, but in the control group, disease signs appeared (7.4%). By day 6, both LAB- and *Bacillus* treatments had a disease incidence of 28.57%, a statistically significant difference compared to the control, which had a disease incidence of 47.61%. At storage day 9, the LDC11 treatment had an incidence of 53.33 and 60% for the BHL21 treatment, which was statistically different from the control (the incidence was 100%). At the end of the storage day, all treatments had an infection incidence of 100%. Gamagae et al. (2003) reported that yeast treatment (*Candida oleophila*) could reduce the anthracnose disease caused by *Colletotrichum gloeosporioides* on papaya (*C. papaya*) fruits in storage. In addition, *Trichosporon asahii* treatment was to inhibit the growth of *C. gloeosporioides* on papaya *in vitro* and *in vivo* test (Hassan et al. 2021). The findings are in agreement with Chavez-Diaz et al. (2019), who reported that cell suspensions (CS) and 'cell–free' supernatants (CFE) from *B. subtilis*, *B. subtilis*, and *B. licheniformis* were very effective in controlling soft rot caused by *Rhizopus stolonifer* in blackberry fruits.

Effect of isolates LDC11 and BHL21 on the disease severity in papaya fruit

The results showed that the disease severity increased in all the treatments during the 12 days of storage. However, disease severity in the two bacterial treatments was significantly ($P<0.05$) lower compared to the control treatment during the storage period (Figure 9.B). By day 3, the disease severity was only observed in the control (13.88%). On days 6 and 9, the highest disease severity was recorded in the control treatment (the disease severity in these periods was 52.38 and 63.33%, respectively). At the end of storage day 12, the highest disease severity was found in the control treatment (94.44%), which was statistically significant ($P<0.05$) compared with the fruits subjected to LAB (80.55%) and *Bacillus* treatment (72.22%). This result is because LAB and *Bacillus* treatment may produce the antifugal compounds (proteinaceous substances, organic acids, and hydrogen peroxide) or lytic enzymes, nutrient and space competition, signal interference and induced systemic resistance (ISR) in plants (Chen et al. 2020; Oirdi et al. 2021) that could be inhibit the growth of fungal mycelium. Therefore, LAB and *Bacillus* treatment could reduce the disease severity at day 12 in compared with the control treatment. Previous studies showed that *Bacillus amyloliquefaciens* treatment had significantly reduce anthracnose disease (disease incidence and severity) on papaya fruit (Osman et al. 2011). In addion, on tomatoes, Hamed et al. (2011) demonstrated the inhibitory effectiveness of using LAB isolated from milk and yogurt to control *Fusarium oxysporum*. The efficiency of utilizing *B. subtilis* CF-3 isolated from yogurt in fighting fungal diseases on peaches and litchi was also demonstrated by research by Gao et al. (2018). These authors demonstrated that *B. cinerea*, *Monilinia fructicola*, *C. gloeosporioides*, *Penicillium expansum*, and *A. alternata* were all susceptible to *B. subtilis* CF-3's antifungal effects in this study.

Weight loss

The results showed that the weight loss of LAB- and *Bacillus*-treated papaya fruits increased during the storage period (Figure 10.A). However, the weight loss for the fruits subjected to the treatment with LAB and *Bacillus* was significantly ($P<0.05$) lower than the control (weight loss at days 3, 6, 9, and 12 were 1.8, 4.5, 6.9, and 9.2%, respectively). The findings align with the study conducted by Kaarunya et al. (2022), who reported that the weight loss of tomato fruit treated with LAB was lower than that of tomato fruit not treated with LAB.

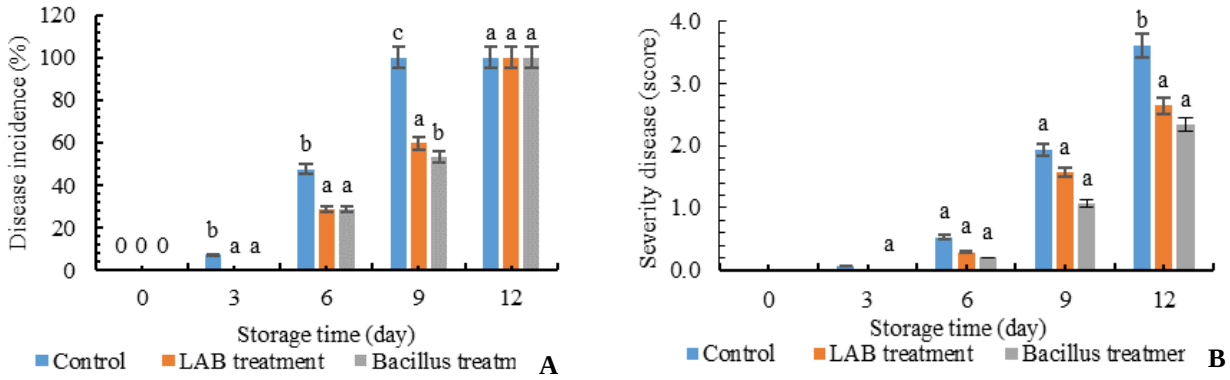

Figure 9. Disease incidence and severity of disease in papaya fruits during storage. Note: Vertical bars represent SE, means followed by different letters are statistically significant ($p<0.05$)

Vitamin C content

The results showed that the vitamin C concentration declined steadily with storage time. In two bacterial treatments, vitamin C was higher than in the control. However, there was no significant difference (P>0.05) between the bacteria-treated-treatment and the control from storage days 0 to 12 (Figure 10.B). At 12 days of storage, the vitamin C content in the control treatment decreased by 85.41%, significantly higher than the two bacteria treatments of 51.27 and 63.66%, respectively. Research by Wang et al. (2010) indicated that *B. subtilis* EXWB1-inoculated melon retained high levels of vitamin C.

Total Soluble Solid (TSS)

The findings showed that the TSS increased in all treatments during the storage days, and the TSS in bacteria-treated fruits was lower than the control treatment (Figure 11.A). However, no statistically significant difference **(**P>0.05**)** in TSS was found between papaya fruits subjected to the bacteria and the control from day 0 to day 12 (Figure 11.A). The TSS in the control treatment was 7.5 °Brix at day 0, which increased until it reached 9.8 °Brix on the last day of storage. TSS in LAB treatment increased from 7.6 at day 0 to 9.4 °Brix at storage day 12. In *Bacillus* treatment, meanwhile, TSS increased from 7.5 at day 0 to 10 °Brix at storage day 12. This result conflicts with a study by Wang et al. (2021), who found that strawberry fruit treated with *B. halotolerans* KLBC XJ-5 sustained higher TSS contents than the control group. Nevertheless, after 4 days at 22°C, there were no statistically significant differences in fruit TSS between the two groups.

Total titratable acid content

The research showed that TA decreased in all treatments during the storage days (Figure 11.B). From storage days 0 to 6, TA in papaya fruits subjected to bacteria was not significantly higher (P>0.05) compared to the control. Meanwhile, TA in the control treatment was not significantly higher than that of the bacteria-supplemented treatments from storage days 9 to 12 (Figure 11.B). According to the study of Wang et al. (2021), strawberry fruits treated with *B. halotolerans* KLBC XJ-5 retained higher TA contents than those in the control group. However, after 4 days at 22°C, there were no statistically significant differences in fruit TA between the two groups.

Firmness

The study showed that the firmness of the three treatments decreased during the storage period (Figure 11.C). It can be seen that there was no significantly difference fruit's firmness between the control and *Bacillus* treatment from day 0 to 12. While, the first 3 days of the storage period, no difference in firmness between the control and LAB treatment. However, from day 6 to 9, the papaya fruit treated with LAB had a lower firmness than the control but in the end of storage, the firmness of LAB treatment had higher than the control. Therefore, throughout this study, it was shown that fruit's firmness was maintained during storage due to two isolates that inhibited the growth of *Collectotrichum*. Pingping et al. (2017) supposed that one of the most crucial factors in assessing whether biocontrol agents can be utilized in the actual postharvest process is the impact of fruit quality. The findings align with the previous study by Wang et al. (2010), who demonstrated that melon fruits treated with *B. subtili*s EXWBl were also firmer than those treated with chlorine dioxide or a preservative for 10 days at 25°C. The research of Yuan et al. (2022) revealed that *B. velezensis* strain P2-1 had no discernible impact on the firmness of apple fruits.

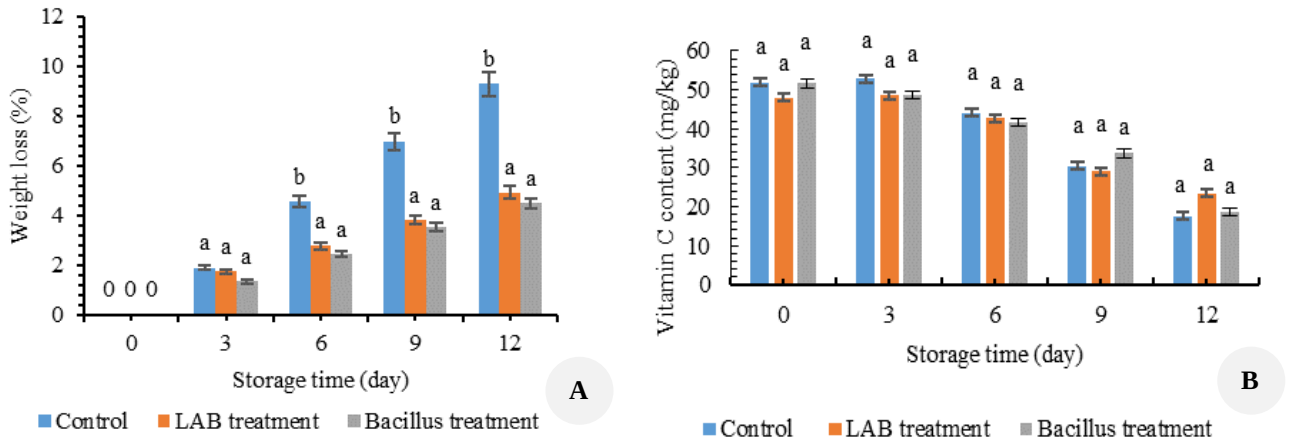

Figure 10. Weight loss and vitamin C content of papaya fruit during storage. Note: Vertical bars represent SE, means followed by different letters are statistically significant (p<0.05)

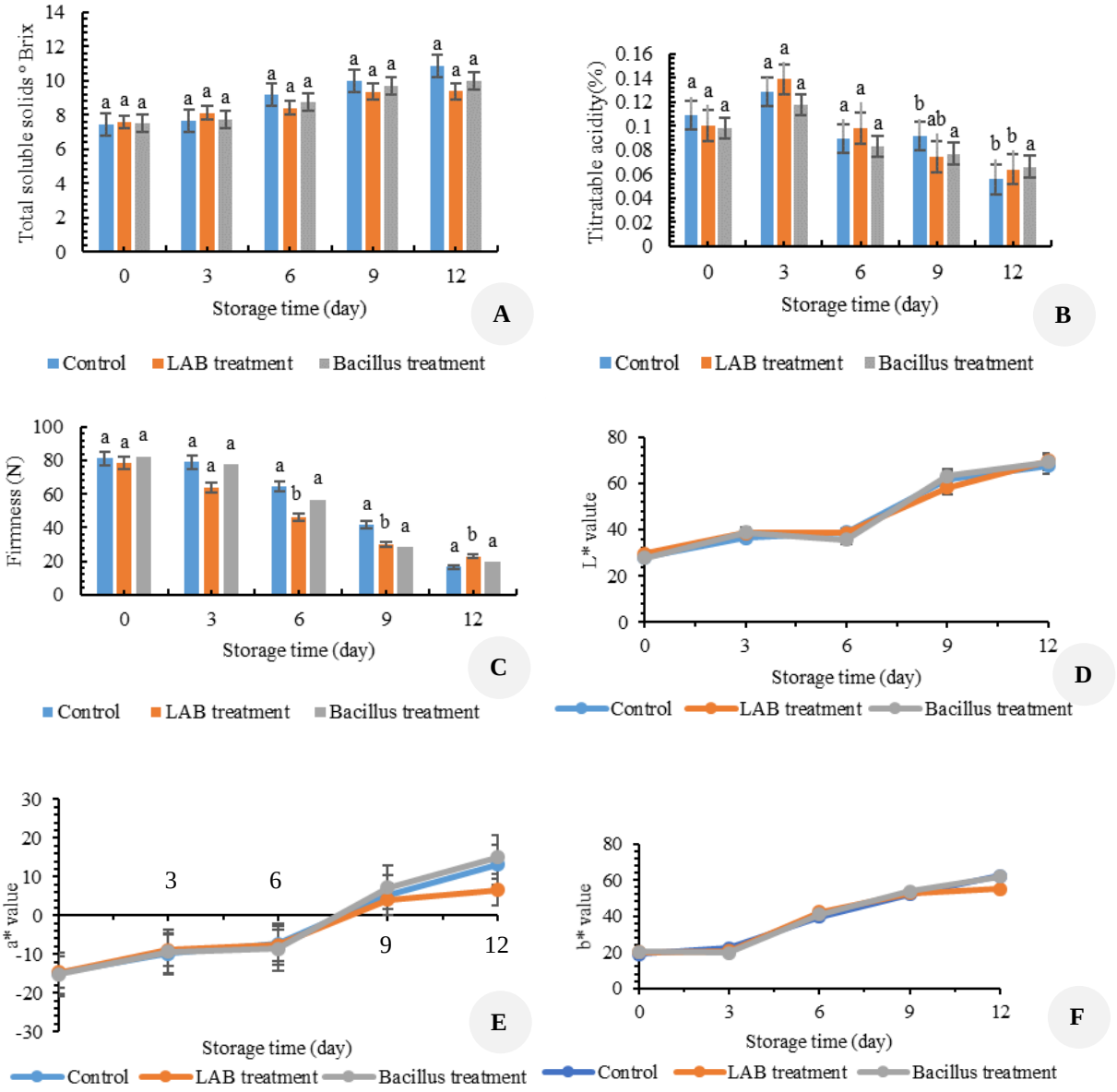

Figure 11. Effect of isolates LDC11 and BHL21 on TSS, TA, and color fruit during the storage period. Note: Vertical bars represent SE, means followed by different letters are statistically significant ($p<0.05$)

Fruit color

The results showed that L* values increased in all three treatments during storage. Although the L* value increased the most in the control treatment, the difference was not statistically significant ($P>0.05$) compared with the two bacteria treatments (Figure 11.D). In this study, the a* values in all three treatments increased, but the a* value in the control treatment increased more; the difference was not statistically significant ($P>0.05$) when compared to the two treatments with bacterial treatment (Figure 11.E). The a* value at day 12 in the controls (LAB- and *Bacillus*-treatment) was 13.21, 6.62, and 15.08, respectively. Meanwhile, the observations showed that the b* values in the three treatments increased, but the b* values in the control treatments increased even more. However, the difference was not statistically significant ($P>0.05$) compared with the two bacteria treatments (Figure 11.F). At day 12, the b* values of the controls, LAB- and *Bacillus*-treatment, were 62.41, 54.98, and 61.96, respectively. This result in agreement with Osman et al. (2011) who reported that *Bacillus amyloliquefaciens* treatment had no effect on the color of postharvest papaya fruit in storage. In this work, two BHL21 and LDC11 isolates had to reduce the anthracnose disease in postharvest papaya fruit and extending the sheflife. However, no significantly impact on the postharvest papaya fruit quality during storage.

In conclusion, the study isolated and identified LAB and *Bacillus* isolates from traditional fermented vegetables and papaya rhizospheric soils with antagonistic activity against *Colletotrichum* spp. In particular, two isolates, LDC11 and BHL21, have the highest antagonistic ability against *Colletotrichum*. At a 10^6 CFU/mL concentration, two isolates, LDC11 and BHL21, reduced disease incidence and severity and weight loss in comparison to the control. However, the LAB and *Bacillus*-treated papaya fruit did not affect the fruit quality such as color changes, firmness, TSS, TA and vitamin C content during storage. Therefore, to improve the quality of postharvest papaya fruits, the effect of isolates BHL21 and LDC11 combined with further treatment such as edible coating or modified atmosphere packaging should be investigated in future researchs.

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ARTICLES FOR FACULTY MEMBERS

EFFECT OF LACTIC ACID BACTERIA AS BIO-PRESERVATION AGAINST SPOILAGE FUNGI FROM PAPAYA FRUIT

Influence of fermentation of pasteurised papaya puree with different lactic acid bacterial strains on quality and bioaccessibility of phenolic compounds during in vitro digestion / Mashitoa, F. M., Akinola, S. A., Manhevi, V. E., Garcia, C., Remize, F., Slabbert, R. M., & Sivakumar, D.

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Influence of Fermentation of Pasteurised Papaya Puree with Different Lactic Acid Bacterial Strains on Quality and Bioaccessibility of Phenolic Compounds during *In Vitro* Digestion

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Abstract: This study describes the impact of utilising different strains of lactic acid bacteria (LAB) for the fermentation of papaya puree and their effect on the quality parameters and bioaccessibility of phenolic compounds during simulated *in vitro* gastrointestinal digestion. Papaya was processed into puree; pasteurised and fermented at 37 °C for 2 days; and stored for 7 days at 4 °C using LAB strains *Lactiplantibacillus plantarum 75* (*L75*D2*; *L75*D7*), *Weissella cibaria 64* (*W64*D2*; *W64*D7*) and *Leuconostoc pseudomesenteroides 56* (*L56*D2*; *L56*D7*), respectively. Non-fermented samples at 0 (*PPD0*), 2 (*PPD2*) and 7 days (*PPD7*) served as controls. pH was reduced with fermentation and was lowest in *L56*D2* (3.03) and *L75*D2* (3.16) after storage. The colour change (ΔE) increased with the fermentation and storage of purees; *L75*D7* showed the highest ΔE (13.8), and its sourness reduced with storage. The fermentation by *W64*D7* and *L75*D7* increased the % recovery of chlorogenic, vanillic, syringic, ellagic, ferulic acids, catechin, epicatechin and quercetin in the intestinal fraction compared to the *L56*D7* and *PPD7*. Fermentation by *W64*D7* and *L75*D7* significantly improved the antioxidant capacity of the dialysed fraction compared to the *L56*D7* or *PPD7*. *L56*D7*-fermented papaya puree showed the highest inhibitory effect of α-glucosidase activity followed by *L75*D7*. *L75*D7* had a significantly higher survival rate. LAB fermentation affected the bioacessibilities of phenolics and was strain dependent. This study recommends the use of *Lpb. plantarum 75* for fermenting papaya puree.

Keywords: postharvest preservation; *Lactobacillus*; antioxidant activity; polyphenols; *in vitro* digestion

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1. Introduction

Regular consumption of fruits and vegetables is important for a healthy lifestyle, and for the reduction in risk factors for non-communicable diseases [1]. Reports from the World Health Organization (WHO) and Food and Agriculture Organization (FAO) [2] of the United Nations in 2004 recommended an intake of 400 g of fruit and vegetable per day. However, the highly perishable nature of fruits and vegetables and lack of cold chain facilities, coupled with the energy cost requirements, limit their availability and shelf life. Therefore, fermentation technology guarantees the availability and safety of fruits and vegetables to the consumers during the off season [3]. Several researchers have reported an improvement in the antioxidants of fruits and vegetables during lactic acid fermentation [4,5].

Carica papaya Linn., belonging to the family *Caricaceae*, known as pawpaw or papaya, is popularly produced and consumed in South America, Asia and Africa [6]. Papaya is a

rich source of carotenes, vitamin C, flavonoids, antioxidants, folate, potassium, magnesium and fibre [6]. LAB fermentation rapidly reduces the pH, thus increasing acidity, which prevents the spoilage of fermented products [7]. Furthermore, fermentation enhances the antioxidant properties through the biotransformation of phenolic compounds by the metabolising LAB strains, resulting in the release of bioactive compounds [8]. However, the potential functionality of a compound depends on the amount that is available after gastrointestinal digestion compared to the original amount before digestion. The pH changes that occur during gastrointestinal digestion phases produce phenolic derivatives that are high in molecular weight, have low solubility and are unavailable for absorption, mainly due to oxidation or polymerisation reactions [9]. Shahid and Peng [10] stated that the interaction with protein, lipid, fibre and hydrolytic enzymes affects the bioaccessibility of phenolic compounds in the intestinal tract. *In vitro* digestion models are widely used by researchers to mimic digestion due to it cost effectiveness [9] and non-ethical clearance requirement. Pavan et al. [11] showed changes in total phenols and antioxidant activity during gastrointestinal digestion before and after the digestion of tropical fruits, such as araticum, papaya and jackfruit, and digestion decreased the levels of total phenols and antioxidant activity in papaya extracts. However, their finding did not show the influence of gastrointestinal digestion on predominant different phenolic components present in papaya juice. Therefore, we hypothesise that the fermentation with LAB increases the total phenol content, different phenolic metabolites and antioxidant capacity in fermented papaya puree after digestion at the dialysis phase, which is available for intestinal absorption.

The total phenolic content in papaya is reportedly 54 mg GAE/100 g fresh weight (FW) [12]; however, the concentrations differ in various cultivars. Gayosso-García Sancho et al. [13] reported the ferulic acid content as 277.49–186.63 mg/100 g dry weight (DW), p-coumaric acid (229.59–135.64 mg/100 g DW) and caffeic acid (175.51–112.89 mg/100 g DW) contents in Maradol papaya from Mexico. Phenolic compounds inhibit α-glucosidase and reduce glucose uptake in the small intestine, through the inhibition of disaccharide digestion [14]. A commercial fermented papaya preparation sold in Japan and the Philippines reportedly showed a significant decrease in plasma glucose levels in type 2 diabetic patients [15]. Therefore, this study was aimed to investigate the effect of LAB fermentation on the quality parameters, changes in major phenolic compounds, α-glucosidase activity and to evaluate the influence of *in vitro* gastrointestinal digestion on phenolic components and antioxidant capacity of the pasteurised and fermented papaya puree.

2. Materials and Methods

2.1. Chemicals

Culture media were purchased from Biokar Diagnostics (Solabia group, Pantin, France) and Conda Laboratories (Madrid, Spain). Reagents were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France) and VWR chemicals (Fontenay-sous-Bois, France). Type VI-B porcine pancreatic α-amylase, type I α-glucosidase from baker's yeast, starch, p-nitrophenyl-β-glucopyranoside (pNPG) and voglibase and other chemicals came from Sigma-Aldrich (Saint-Quentin Fallavier, France).

2.2. Preparation of Fruit Purees

The purchase of fruits was from the local growers in Réunion Island. Purees were prepared by peeling, cutting and blending the fruit pieces. The bottled fruit purees were then pasteurised in an agitating water bath at 80 °C for 15 min and cooled to room temperature (28 °C) for 2 h prior to fermentation.

2.3. Reactivation of LAB Cultures and Fermentation of Fruit Purees

Lactic acid bacteria used in the study were previously isolated from tomatoes (*Lycopersicon esculantum*), papaya (*Carica papaya*) and sliced cabbage (*Brassica oleacera var. capitata*) and genotyped and have been reported as safe [3]. The LAB strains *Leuconostoc pseudome-*

senteroides 56, Weissella cibaria 64 and *Lactiplantibacillus plantarum 75* were reactivated at 30 °C for 48 h in de Mann Rogosa Sharpe (MRS) broth (Biokar Diagnostics, Pantin, France).

An aliquot of 100 µL of each culture was transferred into new 9 mL MRS broth and then incubated for 48 h at 30 °C. Repeating the reactivation step twice was to achieve an active growing condition of the cultures, after which the culture cells were produced anaerobically in MRS broth incubated at 30 °C for 24 h. The LAB cell pellet, obtained after centrifugation at 8000× *g* for 5 min, was washed twice with sterile distilled water. The resulting LAB cells, re-suspended in 20 mL of sterile water, were used as stock of concentrated LAB cultures. The concentrations of the LAB cultures were determined using a spectrophotometric method via the optical density measurements in BMG LABTECH GmbH, SpectroStar Nano, Ortenberg, Germany. A concentrated LAB cell was appropriately diluted to 0.05 McFarland standard concentrations (6 Log CFU/mL) at 660 nm. To 100 g of puree, 1 mL of the LAB culture (6 Log CFU/mL) was inoculated and incubated at 37 °C for 2 days; after this, it was stored for 7 days at 4 °C. The non-fermented purees at 0 (*PPD0*) and 2 days (*PPD2*), and stored at 4 °C for 7 days (*PPD7*), were used as controls. Other treatments included papaya puree fermented with *Leu. pseudomesenteroides 56* for 2 days (*L56*D2*) and stored at 4 °C for 7 days (*L56*D7*), papaya puree fermented with *W. cibaria 64* for 2 days (*W64*D2*) and stored at 4 °C for 7 days (*W64*D7*), and puree fermented with *Lpb. plantarum* 75 for 2 days (*L75*D2*) and stored at 4 °C for 7 days (*L75*D7*). Fermented and non-fermented purees were stored at −20 °C prior to analysis, and the fermentation was performed in triplicate.

2.4. Physicochemical Properties of Fermented and Non-Fermented Papaya Puree

The physicochemical properties of only pasteurised and fermented papaya puree were determined at 0 and 2 days of fermentation, and 7 days of storage. The pH was measured using the EUTECH pH2700 Instruments (EUTECH Instruments, Illinois, IL, USA), while the total soluble solids (TSS) of samples was measured using the ATAGO PAL-3 pocket refractometer (Atago USA Inc., Tokyo, Japan). The obtained refractive index values were recorded in °Brix. The total titratable acidity of samples was determined according to the method of Reddy et al. (2015). The effect of fermentation and storage on the colour of purees was determined using a CM-3500 d spectrophotometer that made use of spectraMagic NX software (Konica Minolta, Konica Minolta Sensing Inc, Tokyo, Japan). The degree of lightness (*L**), red to green component (*a**), and yellow to blue (*b**) colour components of samples were measured. The calculation of the total colour difference (ΔE) was performed according to Managa et al. [16].

2.5. Determination of Microbial Count and Survival of LABs

The evaluation of the total viable count and surviving LAB count of the puree used pour plating techniques [17]; the plating of the serially diluted samples was on appropriate media. For the total fungal (yeast and mould) counts, the plating of the dilutions was on Yeast Extract Glucose Chloramphenicol Agar (YGCA), bacteria count on nutrient agar (NA) and surviving LAB count on MRS agar plates. The NA plates underwent incubation at 37 °C for 24 h, YGCA plates at 27 °C for 5 days, while the incubation of the MRS plates was performed anaerobically at 30 °C for 48 h. The surviving LAB, aerobic bacterial and fungal counts were enumerated as logarithmic colony forming units per gram (Log CFU/g) of sample.

2.6. Organoleptic Properties of Non-Fermented and Fermented Stored Papaya Purees

The sensory evaluation of the puree used a quantitative descriptive analysis technique described by Oliveira et al. [18], with some modifications. The selection of nine trained panellists was from the pool of assessors trained to identify the desired characteristics of the puree. The panellists were composed of healthy male and female research employees. There were two training sections adopted, and the samples were rated using a structured scale ranging from 0 to 6 (absent = 0; 1–2.4 = weak; 2.5–3.9 = moderate; –6 = strong). The assessment of the perception of bright or dark orange colour was performed using ripe

papaya juice (100%) and papaya juice with 1% food grade browning as a reference, respectively. The characteristic aroma of papaya was assessed using a ripe papaya pulp juice (100%), while the characteristics of a viscous food in the mouth (consistency) was assessed using (30%) glucose syrup solution as a reference. The assessment of the perception of acid taste and fermented fruit (sourness) was performed by using a commercial unsweetened yoghurt, while the sweet taste characteristics on the tongue were evaluated using sucrose solution (70%) as a reference. A commercial fresh fermented fruit concentrate was used as a reference to determine the overall acceptability. Coded samples were served chilled in white cups with lids to the panellists in a white light-illuminated cubicle. The means of the attributes were calculated, and the cut-off point was set at 2.5 for the acceptability of attributes.

2.7. Determination of Total Phenolic Content

Total phenolic content was determined according to Fessard et al. [3], using 30 µL of 10-fold diluted sample and 150 µL of Folin-Ciocalteu reagent, and afterwards adding 60 µL of 700 mM Na_2CO_3 and holding in the dark for 1 h. The absorbance at 760 nm was measured (Infinite M200 PRO, Tecan, Mannedorf, Switzerand) and results expressed in milligrams of gallic acid equivalent (GAE).

2.8. Simulated In Vitro Gastrointestinal Digestion

In vitro digestion, to test the bioaccessibility of antioxidant compounds, was carried out on ethanolic extract of fruit purees according to Brodkorb et al. [19], mimicking the gastric, intestinal and dialysis phases. A set of 10 g of fruit puree (fermented and non-fermented, stored for 7 days) was mixed with 16 mL of simulated gastric fluid (SGF). The mixture, held at pH 1.3 by adjusting with 6 M HCl, was incubated with freshly prepared pepsin solution (10 mL solution in 0.1 M HCl), sufficient to generate a 142 mg/mL sample. After 2 h, simulated intestinal fluid (SIF) was added to the gastric solution and the pH maintained at 7.0 with 5 M NaOH before adding freshly prepared pancreatin-bile salt solution (39.2 mL of pancreatin + 6 mL of bile salts solution in 1 M $NaHCO_3$) to produce a sample of 8.375 mg/mL. The mixture was held for 2 h at 37 °C with shaking at 100 rpm. The collection of the samples (10 mL) was carried out after the intestinal phase, with the remaining used for the dialysis phase. The sample remained at −20 °C to stop intestinal digestion. For dialysis, a dialysis bag (10 cm max, mw cut-off 10 kda) was filled with 5.5 mL NaCl (0.9%) and 5.5 mL $NaHCO_3$ (0.5 M) placed in a beaker filled with SIF (70 mL) and incubated for 45 min at 37 °C, with shaking at 100 rpm. For analysis purposes, a 10 mL sample collected from the dialysis bag was lyophilised.

2.9. Determination of FRAP Activity

Total antioxidant scavenging activity was determined according to Managa et al. [16] using 0.2 g freeze-dried fruit puree extracted using 2 mL of sodium acetate buffer (pH 3.6). An amount of 220 µL of FRAP reagent solution was placed on a microplate (10 mmol/L TPTZ (2,4,6-tris (2-pyridyl)-1,3,5-triazine) acidified with concentrated HCl and 20 mmol/L $FeCl_3$), followed by 15 µL of the homogenised puree extract. The absorbance measurement was performed at 593 nm (Spectrophotometer BMG LABTECH GmbH, SpectroStar Nano, Ortenberg, Germany). The reducing antioxidant power was expressed in Trolox µmol TEAC/100 g DW.

2.10. Effect of Digestion on the Phenolic Profile of Fermented and Non-Fermented Papaya Purees

Extraction and analysis of phenolics in fermented and non-fermented digested purees were performed according to the method of Palafox-Carlos et al. [20]. Digesta from fermented and non-fermented purees were freeze-dried (0.25 g) and homogenised into 10 mL of 80% methanol containing BHT (1 g/L); then, 5 mL of 6 M HCL was homogenised using a BV1000 vortex mixer (Benchmark Scientific Inc., Sayreville, NJ, USA) and the mixture stirred carefully. A 2510 model ultrasonic bath (Branson, LabFriend Pty Ltd., North Sydney,

NSW, Australia) sonicated the mixture for 30 min at 70 °C, then centrifuged it at 10,000 rpm for 15 min at 4 °C using a Hermle centrifuge (Model Hermle Z326k, Hermle Labortechnik GmbH, Wehingen, Germany). The collected supernatants were filtered through a 0.22 µm PTFE syringe filter (Grafiltech). The resulting filtrate was injected and analysed in the HPLC/UV-DAD system using a Shimadzu Prominence-i-LC-2030C 3D, Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), coupled to a diode array detector for HPLC analysis. Chromatographic separation was achieved using a Shim-pack Gist C18 5 µm, 4.6 × 250 mm reverse phase column with gradient elution at 30 °C, using a 10 µL injection volume. The mobile phase consisted of 6% glacial acetic acid (solvent A) and 75% acetonitrile containing 5% glacial acetic acid (solvent B). The elution gradient was 0–100% (B) for 30 min, kept for 5 min at 100% (B), then returned to 0% (B) for 3 min at a flow rate of 0.6 mL/min. The detection of analytes was performed at 280 nm, and the compounds identified based on a combination of retention time and spectral matching based on standards.

2.11. Determination of α-Glucosidase Inhibition

For the effect of fermentation on the antidiabetic activity of fermented and non-fermented purees, alpha-glucosidase assay was performed using samples obtained on day 7 [21]. Purees were homogenised with sodium phosphate buffer (0.1 M) and dilutions $\frac{1}{2}$, 1/5, 1/10 and 1/20 obtained. An aliquot of 62 µL of sodium phosphate buffer, 50 µL of enzyme and 62 µL of inhibitor sample underwent mixing in a 96-well plate, and incubated for 2 min at 37 °C under shaking. To the wells, 25 µL of substrate solution was added and absorbance measured at 405 nm ((Infinite M200 PRO, Tecan, Mannedorf, Switzerand). The activity and percentage of enzyme inhibition in fruits extracts were determined using Equation (1) and Equation (2), respectively:

$$\text{OD test}_{405} = \text{OD sample} - \text{OD negative control} \quad (1)$$

$$\%\ \text{Glucosidase inhibition} = 100\% - \text{activity of OD test} \quad (2)$$

2.12. Statistical Analysis

This study used a completely randomised design with five replicates per treatment. The fermentation experiments were performed twice to ensure the reliability of data. One-way analysis of variance (ANOVA) tested the significant differences between the means. Means were compared among the treatments by the least significant difference (LSD) test, at $p < 0.05$, using the Genstat statistical programme for Windows 13th Edition, 2010 (VSN International Hempstead, Hertfordshire, UK).

3. Results and Discussion

3.1. Physicochemical Properties of LAB-Fermented Papaya Purees

Lactic acid bacteria have the ability to break down carbohydrates into organic acids [3], which could help in food preservation and enhance the safety of food. The pH of fermented and non-fermented papaya puree was in the range of 3.03 to 5.08 (Table 1). The pH values of purees reduced with fermentation after 2 days compared to the non-fermented sample (5.08). The highest reduction in the pH was obtained in *L56*D2* (3.03) and was not significantly different to *L75*D2* (3.16) fermented for 2 days, while the non-fermented at day zero had the highest pH (5.08). Moreover, after storage for 7 days, there was a significant increase in the pH of the fermented purees, except the control ($p \leq 0.05$), relative to values at 2 days of fermentation. The increase in pH after 7 days of storage might have been caused by the poor survival of the LAB due to inappropriate conditions for growth during storage at 4 °C, which could have enabled the growth and release of some yeast metabolites that could raise the pH. The titratable acidity contents of purees ranged from 0.59 to 0.94 mg/mL lactic acid and increased with fermentation relative to the non-fermented sample. The titratable acidity was highest in *L75*D2* (0.94 mg/mL) and lowest in the non-fermented puree (0.59 mg/mL) at day zero. The fermented samples were

significantly different to the non-fermented samples ($p \leq 0.05$), while the non-fermented samples at day 0 and 2 were not significantly different to each other ($p > 0.05$). The decrease in the pH with an increase in titratable acidity contents after 2 days of fermentation could be due to the activity of inoculated LABs in converting carbohydrate substrates in the purees into organic acid after two days of fermentation. Ayed et al. [22] reported a similar observation of pH decrease after fermentation in red grape juice.

Table 1. Changes in pH and total soluble solids (°Brix) of non-fermented and fermented papaya puree.

Fruit Puree	pH	Titratable Acidity (mg/mL)	Total Soluble Solids (Brix°)
PPD0	5.08 ± 0.01^{a}	0.59 ± 0.04^{d}	$8.03 \pm 0.06^{a,b}$
PPD2	$4.99 \pm 0.01^{a,b}$	0.63 ± 0.02^{d}	$8.07 \pm 0.06^{a,b}$
PPD7	4.36 ± 0.01^{b}	0.68 ± 0.02^{c}	8.50 ± 0.02^{a}
*L56*D2*	3.03 ± 0.01^{d}	0.76 ± 0.02^{b}	6.87 ± 0.06^{d}
*L56*D7*	3.88 ± 0.01^{c}	0.73 ± 0.02^{b}	8.60 ± 0.02^{a}
*W64*D2*	3.76 ± 0.01^{c}	$0.80 \pm 0.02^{a,b}$	7.13 ± 0.06^{c}
*W64*D7*	4.09 ± 0.01^{b}	0.71 ± 0.02^{b}	7.80 ± 0.17^{b}
*L75*D2*	3.16 ± 0.01^{d}	0.94 ± 0.04^{a}	6.83 ± 0.06^{d}
*L75*D7*	3.36 ± 0.01^{c}	$0.84 \pm 0.04^{a,b}$	7.13 ± 0.07^{c}

Values are mean± standard error of means; means followed by a different letter within the column are significantly different ($p \leq 0.05$).

Key: *PPD0* = non-fermented papaya puree at 0 days; *PPD2* = non-fermented papaya puree at day 2; *L56*D2* = papaya puree fermented with *Leu. pseudomesenteroides* 56 for 2 days; *W64*D2* = papaya puree fermented with *W. cibaria 64* for 2 days; *L75*D2* = papaya puree fermented with *Lpb. plantarum 75* for 2 d; *PPD7* = non-fermented papaya puree stored at 4 °C for 7 d; *L56*D7* = papaya puree fermented with *Leu. pseudomesenteroides 56* stored at 4 °C for 7 d; *W64*D7* = papaya puree fermented with *W. cibaria 64* stored at 4 °C for 7 d; *L75*D7* = papaya puree fermented with *Lpb. plantarum 75* and stored at 4 °C for 7 d.

The titratable acidity contents of purees decreased slightly during storage for 7 days, thus signifying a decline in the fermentative activity of the inoculated LABs in the purees due to the possible death of some LAB cells during storage. The increase in pH during storage, as observed in the fermented purees, could be due to a decline in active LAB counts, which corroborates the decrease in puree acidity after storage. The reduced acidity could have enhanced the activities of some competing bacteria and fungi, which utilise organic acids as a carbon source, thereby producing metabolites that lower the pH of the purees [17]. Similar to the reduction in pH observed in this study, LAB fermented emmer -based beverages fortified with fruit juices have been reported to have lower pH after fermentation [23]. The higher degree of acidity characterised by low pH, as observed in *L75*D2* at day 2 of fermentation and 7 days of storage, is suggestive of *Lpb. plantarum* being a strong hetero-fermenter that could survive at low pH and temperature.

The TSS content of the fermented and non-fermented purees ranged from 6.83 to 8.60 °Brix (Table 1) and was highest in *L56*D7* and lowest in *L75*D2*. The TSS content was significantly reduced after fermentation for 2 days compared to the non-fermented sample ($p \leq 0.05$). However, *L56*D2* and *L75*D2* were not significantly different from each other, in line with the control at day 0 and 2 ($p > 0.05$). The reduction in the TSS contents of the fermented purees compared to the non-fermented purees could be due to the metabolic activities of inoculated LAB cultures that break down, reducing sugars into organic acid, confirmed by a reduction in the pH of the purees. The reduction in the TSS content after fermentation is in agreement with that obtained by Soibam et al. [24] in fermented sugarcane and beet juice. The TSS contents of purees increased during storage for 7 days. Samples *L56*D7* (8.60 °Brix) and non-fermented (8.50 °Brix) puree were not significantly different ($p > 0.05$) from each other after 7 days of storage, in line with samples *L75*D7* and *W64*D2* (7.13 °Brix). The increase in the TSS content during storage could be due to the hydrolysis of carbohydrates into reducing sugars, thus supporting the previous

reports of an increase in the total soluble solid of stored kinnow juice [25] and carrot–orange juice [26].

3.2. Effect of Fermentation and Storage on Colour Characteristics of Papaya Puree

The effect of fermentation and storage on the colour characteristics of papaya puree is presented in Table 2. The luminosity of fermented and non-fermented puree ranged from 29.96 to 43.21 and was highest in the non-fermented puree prior to fermentation, and lowest in *L75*D7* papaya puree stored for 7 days. After fermentation and storage at cold temperature (4 °C), the luminosity (*L**) of the puree reduced relative to the control. The purees fermented for 2 days were significantly different to the non-fermented purees at day 2 ($p \leq 0.05$). The *L56*D2* and *W64*D2* were not significantly different ($p > 0.05$), while *L75*D2* significantly differed to others in terms of luminosity ($p \leq 0.05$). The type of fermenting LAB cultures significantly influenced the colour parameters of the puree. Contrary to that obtained in other treatments, *W64*D7* had increased lightness upon storage for 7 days, thus suggesting the potential ability of *W. cibaria 64* to inhibit enzymatic oxidation in samples.

Table 2. Colour characteristics of fermented and non-fermented papaya puree.

Fruit Puree	*L**	*a**	*b**	Δ*E*
PPD0	43.21 ± 0.01^{a}	23.15 ± 0.01^{c}	$52.76 \pm 0.01^{b,c}$	
PPD2	35.07 ± 0.06^{b}	24.48 ± 0.01^{b}	58.02 ± 0.01^{a}	9.8 ± 0.5^{g}
PPD7	32.43 ± 0.01^{d}	$25.62 \pm 0.01^{a,b}$	54.42 ± 0.01^{b}	11.2 ± 1.0^{d}
*L56*D2*	33.91 ± 0.08^{c}	23.87 ± 0.03^{c}	52.33 ± 0.01^{c}	9.3 ± 0.1^{h}
*L56*D7*	32.13 ± 0.01^{d}	$25.33 \pm 0.01^{a,b}$	53.86 ± 0.01^{b}	11.3 ± 1.0^{c}
*W64*D2*	32.99 ± 0.02^{c}	24.97 ± 0.02^{b}	$53.13 \pm 0.15^{b,c}$	10.4 ± 0.1^{e}
*W64*D7*	33.65 ± 0.01^{c}	24.35 ± 0.02^{b}	$55.72 \pm 0.01^{a,b}$	10.1 ± 0.7^{f}
*L75*D2*	$31.29 \pm 0.01^{d,e}$	24.97 ± 0.02^{b}	50.62 ± 0.01^{d}	12.3 ± 2.3^{b}
*L75*D7*	29.96 ± 0.05^{e}	26.38 ± 0.02^{a}	50.61 ± 0.01^{d}	13.8 ± 0.4^{a}

Values are mean± standard error of means; means followed by a different letter within the column are significantly different ($p \leq 0.05$). *L** = degree of lightness; a* = red to green component; b* = yellow to blue; Δ*E* = total colour difference.

The redness to greenness characteristics (*a**) of the puree ranged from 23.15 to 26.38. The degree of redness to greenness of the puree did not significantly increase with fermentation and storage and was highest in *L75*D7* (26.38). However, *a** in *L56*D2* and *W64*D7* increased after fermentation and storage for 7 days, respectively, while *L56*D2* was not significantly different to the control before fermentation ($p > 0.05$). The yellow to blue components of the puree was in the range 50.61–58.02 and was highest in the non-fermented sample. At day 2 of fermentation, the *b** component of fermented samples was significantly different to the control, except *W64*D2*, while upon storage, samples were not significantly different to the control, except *L75*D7*. The decreased luminosity of the puree and high colour change in stored fermented papaya puree could be due to an enzymatic oxidation caused by the reduced fermentation and acidity in the puree during storage. The *b** colour coordinate related to the yellow colour of the puree used for the calculation of Δ*E* relates to the colour change.

The Δ*E* relates to the colour change, and is associated with the rate of enzymatic browning in fruit juices and purees [26]. The Δ*E* of the samples ranged from 9.8 to 13.8. The colour change in the puree was lowest in *L56*D2* and highest in *L75*D7*. The Δ*E* significantly increased by fermentation and storage ($p \leq 0.05$); therefore, fermenting with different types of LAB strains and storage at a cold temperature influenced the Δ*E* in papaya puree. There was a significant colour change in papaya puree, as its Δ*E* values were greater than two. The colour change in fruit and juices correlate with the enzymatic activities of polyphenolic oxidase [27]. Hence, the high Δ*E* values in stored fermented purees could be due to an auto-oxidation of polyphenolic compounds [28].

Key: *PPD0* = non-fermented papaya puree at 0 days; *PPD2* = non-fermented papaya puree at 2 d; *L56*D2* = papaya puree fermented with *Leu. pseudomesenteroides 56* for 2 d;

*W64*D2* = papaya puree fermented with *W. cibaria 64* for 2 d; *L75*D2* = papaya puree fermented with *Lpb. plantarum 75* for 2 d; *PPD7* = non-fermented papaya puree stored at 4 °C for 7 d; *L56*D7* = papaya puree fermented with *Leu. pseudomesenteroides 56* stored at 4 °C for 7 d; *W64*D7* = papaya puree fermented with *W.cibaria 64* stored at 4 °C for 7 d; *L75*D7* = papaya puree fermented with *Lpb. plantarum 75* and stored at 4 °C for 7 d.

3.3. Survival of LABs in Papaya Purees after Fermentation and Storage

The surviving LAB counts in fermented and non-fermented purees ranged from 0.92 to 9.25 Log CFU/g in puree (Figure 1). As expected, there was a significant increase in the LAB count of purees fermented for 2 days, while upon storage for 7 days, there was a significant decrease, except in *L75*D7*, which was more stable after storage ($p \leq 0.05$). Samples *L75*D2* (9.25 Log CFU/g) and *L75*D7* (9.24 Log CFU/g) were significantly higher than other fermented and non-fermented samples and were not significantly different from one another ($p \leq 0.05$). This suggests the stability and survival of *Lpb. plantarum*-fermented papaya purees at acidic condition after storage for 7 days. *Lpb. plantarum* prefers glucose and lactose as a carbon source and can easily adapt to different conditions [29], which accounts for its versatile use in fermentation. The stability of *L75*D7* puree suggests its potential to be used as functional food (probiotics) that can help to manage dysbiosis in the gastrointestinal tract. *Lpb. plantarum* produces antimicrobial compounds, such as plantaricin, which can inhibit the growth of spoilage and pathogenic microorganisms [30]. At day 2 of fermentation, fermented purees were significantly different from others and the non-fermented samples; however, after storage, *W64*D2* was not significantly different to *L56*D7*. The surviving LAB counts significantly decreased after storage, except in *L75*D7*; this might be associated with the inactivation or death of some LAB cultures due to unfavourable growing conditions during storage. The survival of *Lpb. plantarum* in the puree after storage supports the finding of Srisukchayakul et al. [31] on the acid tolerance of *Lpb. plantarum* in fruit juices stored in refrigerated conditions. The highest LAB count decrease in fermented purees was in *W64*D7*. Thus, *W. cibaria 64* culture might not be able to survive cold storage (4 °C) for 7 days unlike the *Lpb. plantarum*-fermented papaya puree. The variation in the LAB cell survival of fermented mango puree after storage could be due to the unique characteristics of individual LAB cultures used in the study. The fungal counts (1–3 Log CFU/g) and total viable bacteria (data not shown) were within the acceptable limits for fruit juices [32].

Values are the mean ± standard deviation, and means followed by a different letter within the row are significantly different ($p \leq 0.05$). Key: *PPD0*: non-fermented papaya puree at 0 days; *PPD2*: non-fermented papaya puree at day 2; *L56*D2*: papaya puree fermented with *Leu. pseudomesenteroides* 56 for 2 days; *W64*D2*: papaya puree fermented with *W. cibaria* 64 for 2 days; *L75*D*: papaya puree fermented with *Lpb. plantarum* 75 for 2 days; *PPD7*: non-fermented papaya puree stored at 4 °C for 7 d; *L56*D7*: papaya puree fermented with *Leu. pseudomesenteroides* 56 stored at 4 °C for 7 d; *W64*D7*: papaya puree fermented with *W. cibaria* 64 stored at 4 °C for 7 d; *L75*D7*: papaya puree fermented with *Lpb. plantarum* 75 and stored at 4 °C for 7 d; CFU/g: colony forming units per gram of samples.

3.4. Organoleptic Properties of Fermented and Non-Fermented Papaya Puree

The sensory properties of fermented and non-fermented papaya puree are presented in Figure 2. The colour perception ranged from a dull orange (1.67) to a strong bright orange colour (5.33) and was highest in the non-fermented samples at day zero. Similarly, the aroma attributes ranged from weak aroma (2.27) to strong aroma (5.07) perception and was highest in the non-fermented samples at day zero. The perception of the bright orange colour of papaya significantly decreased with storage and was influenced by the type of LAB fermenting the purees ($p \leq 0.05$). After storage, sample *L56*D7* was not significantly different to the non-fermented puree at day 7 in terms of colour and aroma, while a contrary perception was made in *W64*D7* and *L75*D7* ($p > 0.05$). The decrease in lightness could be due to browning caused by an auto-oxidation of the poly-phenolic compounds in the

samples due to possible exposure to metal ions [28]. The lower rating in flavour, especially in *Lpb. plantarum* fermented samples, could be due to the fact that the cultures are known as fermentative heterolactic microorgansims that exclusively produce lactic acids [33], unlike *Leu. pseudomesenteroides* and *W. cibaria,* which are able to produce flavour compounds aside from organic acids during fermentation.

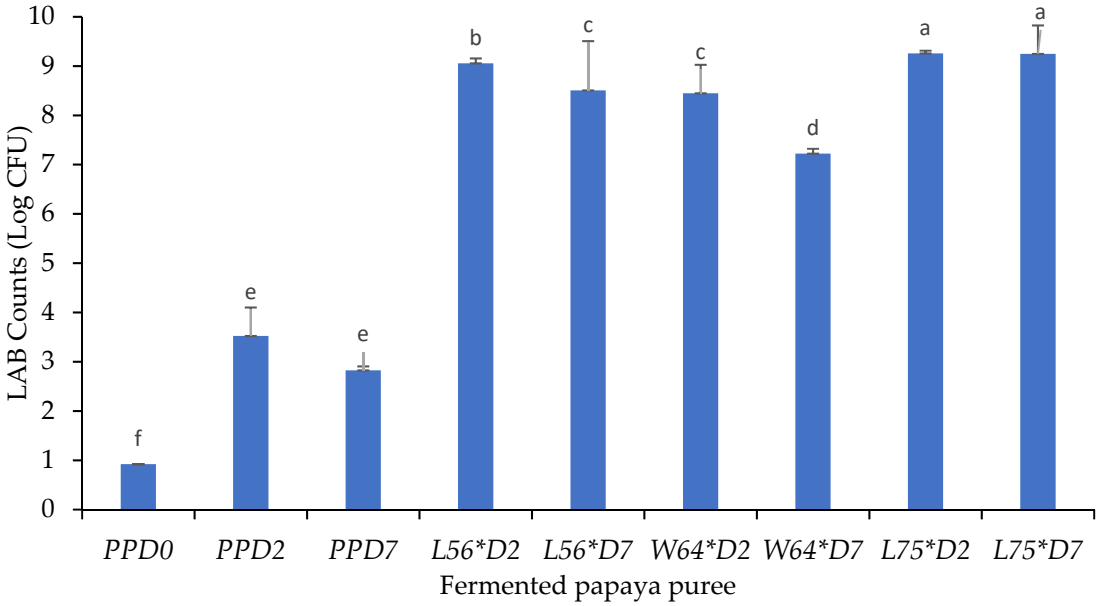

Figure 1. Surviving lactic acid bacteria counts in papaya puree. Bar with different letters are significantly different are significantly different at $p < 0.05$.

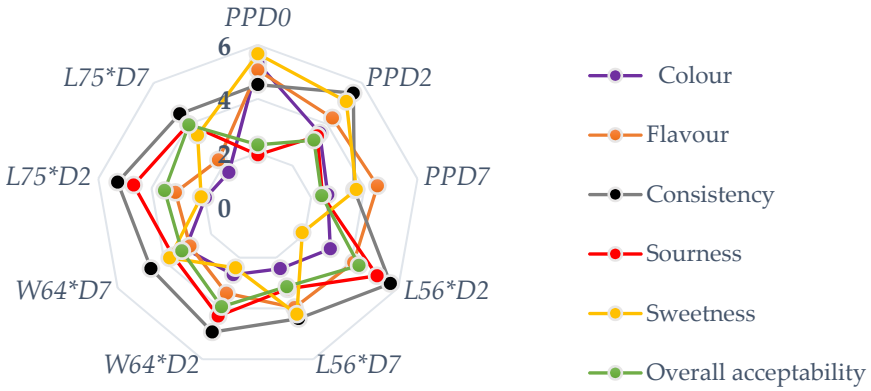

Figure 2. Sensory attributes of pasteurised and fermented papaya puree.

The consistency scores of the product ranged from moderate (3.63) to strong (5.67) and were highest in *L56*D2* and lowest in *PPD7* puree stored for 7 days. The sourness describes the acid taste of the samples, and its perception ranged from a weak (1.93) to strong (5.10) acid taste. The perception of sour taste increased with fermentation but decreased with storage, thus supporting the lower pH in samples after fermentation and the upward increase in pH during storage. The sourness was highest in *L56*D2*; however, it was not significantly different to *L75*D2* and *W64*D2* after fermentation ($p > 0.05$), but differed to the control (*PPD0*, *PPD2*, *PPD7*). Sweetness is the perception of a sweet taste, and it is desirable in some foods, such as purees or juices. The sweetness ranged from weak (1.90) to strong (5.67) sucrose perception and was highest in the non-fermented samples at the start of the experiment (*PPD0*). The sweetness decreased with fermentation and increased during storage. This agrees with the observation made in this study on the decrease in

TSS during fermentation and its slight increase after storage for 7 days. The increase in the sweet taste could have been a product of the de-polymerisation of polysaccharides or other complex carbohydrates in the papaya puree during storage, thus supporting the assertion that slight fermentation could proceed during cold storage, as earlier reported by Managa et al. [34]. The overall acceptability of the fermented and non-fermented stored puree was in the range of 2.30–4.33 (weak to strong) in the product. The highest acceptability was obtained in *L56*D2* but was not significantly different to *L75*D7* (3.97) stored for 7 days, which was slightly different to *L56*D7* and *W64*D7*. Hence, stored puree fermented with *Lpb. plantarum* could deliver a probiotic and nutrient-dense and acceptable product.

Key: *PPD0* = non-fermented papaya puree at 0 days; *PPD2* = non-fermented papaya puree at day 2; *L56*D2* = papaya puree fermented with *Leu. pseudomesenteroides* 56 for 2 days; *W64*D2* = papaya puree fermented with *W. cibaria 64* for 2 days; *L75*D2* = papaya puree fermented with *Lpb. plantarum 75* for 2 days; *PPD7* = non-fermented papaya puree stored at 4 °C for 7 d; *L56*D7* = papaya puree fermented with *Leu. pseudomesenteroides 56* stored at 4 °C for 7 d; *W64*D7* = papaya puree fermented with *W. cibaria 64* stored at 4 °C for 7 d; *L75*D7* = papaya puree fermented with *Lpb. plantarum 75* and stored at 4 °C for 7 d.

3.5. Changes in Phenolic Compounds and Antioxidant Power in Papaya Puree after Fermentation and Storage

The total phenol content and antioxidant capacity and concentration of different phenolic compounds in fresh papaya puree at day 0, non-fermented and LAB fermented papaya puree stored for 7 d at 4 °C are shown in Table 3.

Table 3. Changes in total phenols, antioxidant capacity and phenolic compounds of stored non-fermented and fermented papaya purees.

Parameters	*PPD0*	*PPD7*	*L56*D7*	*W64*D7*	*L75*D7*
Total phenol (mg GAE/100 g FW)	303.9 ± 0.7 ^{e}	408.7 ± 0.8 ^{d}	451.0 ± 0.6 ^{c}	467.1 ± 0.5 ^{b}	475.1 ± 1.9 ^{a}
FRAP (µmol TEAC/100 g FW)	1.4 ± 0.2 ^{c}	2.0 ± 0.3 ^{b}	2.0 ± 0.3 ^{b}	2.7 ± 0.5 ^{a}	2.8 ± 0.2 ^{a}
Phenolic compounds (mg/kg)					
Gallic acid	4.4 ± 1.0	6.7± 1.5	5.6 ± 0.1	2.9 ± 0.2	6.4 ± 0.5
Gallocatechin gallate	581.4 ± 2.7 ^{a}	564.2 ± 1.8 ^{a}	218.9 ± 2.4 ^{d}	462.5 ± 9.8 ^{b}	330.4 ± 11.0 ^{b}
Protocatechuic acid	19.4 ± 0.4 ^{b}	19.3± 0.6 ^{b}	44.4 ± 2.0 ^{a}	19.3 ± 0.8 ^{b}	17.4 ± 0.1 ^{b}
Catechin	14.21 ± 2.3 ^{c}	14.7 ± 1.5 ^{c}	66.1 ± 2.7 ^{a}	58.2 ± 0.7 ^{b}	51.7 ± 1.3 ^{b}
Epicatechin	7.7 ± 0.9 ^{b}	6.2 ± 1.2 ^{b,c}	16.9 ± 0.7 ^{a}	7.7 ± 1.1 ^{b}	5.4 ± 0.4 ^{c}
Chlorogenic acid	19.9 ± 0.1 ^{a,b}	17.2± 0.4 ^{b}	17.7 ± 0.2 ^{b}	1.3 ± 0.8 ^{c}	1.7 ± 0.2 ^{c}
Vanillic acid	5.6 ± 0.6 ^{a}	4.5± 0.8 ^{a}	4.5 ± 0.4 ^{a}	2.5 ± 0.5 ^{b}	2.5 ± 1.1 ^{b}
Syringic acid	4.3 ± 0.1	4.9± 0.1	7.2 ± 0.4	2.5 ± 0.1 ^{e,f}	4.8 ± 1.2 **
Ellagic acid	3.9 ± 2.0 ^{c}	5.4± 1.1 ^{b}	6.2 ± 1.5 ^{b}	3.1 ± 0.2 ^{c}	12.4 ± 0.2 ^{a}
Quercetin	104.9 ± 0.1 ^{a}	103.8± 16.3 ^{a}	38.1 ± 1.5 ^{c}	52.8 ± 5.0 ^{b}	57.7 ± 2.3 ^{b}
p-Coumaric acid	26.9 ± 0.2 ^{b}	23.7± 2.5 ^{b}	37.4 ± 3.2 ^{a}	17.3 ± 0.8 ^{c}	25.4 ± 0.8 ^{b}
Ferulic acid	15.5 ± 0.2 ^{b}	12.6± 0.1 ^{d}	20.5 ± 0.1 ^{a}	13.5 ± 0.2 ^{c,d}	14.3 ± 0.3 ^{b,c}

Values are mean ± standard deviation, and means followed by a different letter within the row are significantly different ($p \leq 0.05$); values within the brackets show % increase or reduction in respective phenolic compounds. Key: *PPD0* = non-fermented papaya puree at 0 days; *PPD7*: non-fermented papaya puree stored at 4 °C for 7 d; *L56*D7*: papaya puree fermented with *Leu. pseudomesenteroides* 56 stored at 4 °C for 7 d; *W64*D7*: papaya puree fermented with *W. cibaria* 64 stored at 4 °C for 7 d; *L75*D7*: papaya puree fermented with *Lpb. plantarum* 75 and stored at 4 °C for 7 d.

The non-fermented papaya puree at 0 d contained the lowest TPC (303.9 mg/100 g DW), while *L75*D7* was the highest (475.1 mg/100 g DW) compared to the non-fermented and fermented purees stored at 4 °C for 7 d. The *L56*D7* was the lowest with regard to total phenol content (451.0 mg/100 g DW) among the other fermented purees (*W64*D7* and *L75*D7*). There was an increase in the total phenol content of lactic acid in fermented plant-based food [35]. Lactic acid bacteria, such as *Lpb. plantarum*, reportedly hold the ability to remove sugar moieties and hydrolysed galloyl moieties from phenolic compounds during fermentation [36]. The higher total phenol content in *Lpb. plantarum*-fermented papaya puree corroborates the report in *Lpb. plantarum*-fermented blueberry juice [37] and could be

attributable to both the hydrolysis of glucosides to aglycones and possibly the production of esterases to which hydrolyses glycosides ester bonds, which could aid the release of insoluble bound and conjugated phenolic compounds [38] in the purees.

The *Leu. pseudomesenteroides 56* could have participated in the partial conversion of simple phenolic compounds and depolymerisation of large molecular weight phenols in papaya puree [38]. To avoid the overestimation of the total phenol content through the spectrophotometric method, there were investigations performed on the changes in individual phenolic compounds during fermentation. The non-fermented puree at 0 d, and fermented papaya puree stored at 4 °C for 7 d contained gallocatechin gallate, gallic acid, protocatechuic acid, vanillic acid, syringic acid, ellagic acid, chlorogenic acid, catechin, epicatechin, quercetin, p-coumaric acid and ferulic acid. Gallocatechin gallate was the most abundant phenolic compound in the non-fermented puree at 0 (581.4 mg/kg) and 7 days of storage (564.2 mg/kg). Gallocatechin gallate reduced significantly ($p < 0.05$) during the fermentation and storage of purees at 4 °C for 7 d. *L56*D7* puree had a 62.34% reduction in gallocatechin gallate compared to the non-fermented puree (*PPD0*). Contrary to that observed in gallocatechin gallate, an increasing trend was observed with catechin after fermentation, with *L56*D7* having a significantly ($p \leq 0.05$) higher concentration (66.1 mg/kg). However, the observation suggests that *Leu. pseudomesenteroides 56* could biotransform gallocatechin gallate to corresponding catechins by its esterase enzymes. The stability of catechins is pH dependent, and they are stable in acidic solution during lactic acid fermentation due to the production of lactic acid [39]; hence, the higher concentration of catechin in *L56*D2* could be due to its high acidic condition, thus resulting in the stabilisation of catechin and epicatechins. Furthermore, the disintegration of the cell wall could have further favoured the extraction of the catechins into the puree [40]. Sample *L56*D7* had the highest protocatechuic acid, p-coumaric acid and ferulic acid concentrations compared to the other fermented and non-fermented purees at 0 and 7 days. The increase in the phenolic acids in the other LAB-fermented purees could be due to the mobilisation of the bound phenolics to a free state via the enzymatic hydrolysis that occurs during fermentation, which could increase their bioavailability [41]. Additionally, *L56*D7* and *L75*D7* showed a significant ($p < 0.05$) increase in vanillic and ellagic acid, respectively. The hydrolysis of ellagitannins during fermentation could have freed and increased the ellagic acid content [42]. Rodríguez et al. [43] reported the ability of LABs to decarboxylate and convert p-coumaric acids to their corresponding vinyl derivatives, which could account for the decrease in p-coumaric acid concentrations in *W64*D7* and *L75*D7*. Contrary to a four-fold increase in quercetin during fermentation initiated by *Lpb. plantarum C2* in Myrtle berries [44], quercetin concentration declined during fermentation in papaya puree. Therefore, the changes in the concentration of phenolic compounds during biotrasformation and metabolic activity depend on the type of LAB strain, and the enzyme systems involved in fermentation, nutrient composition and intrinsic factors of fruit [45].

3.6. Effect of LAB-Fermented Papaya Puree on In Vitro α-Glucosidase Inhibition Activity

The α-glucosidase inhibitory activity of stored fermented and non-fermented papaya purees is presented in Figure 3. The percentage α-glucosidase inhibitory activity ranged from 0% to 37%, of which L*56*D7* (37%) was significantly ($p < 0.05$) higher than L*75*D7* (17%), while other samples had no α-glucosidase inhibitory activity and were comparable to the non-fermented purees at 0 and 7 days. The higher concentrations of protocatechuic acid, catechin, epicatechin, caffeic acid, p-coumaric acid and ferulic acid in L*56*D7* could have contributed towards the inhibition of α-glucosidase activity. Protocatechuic acid ($r = 0.99$, $p < 0.05$), catechin ($r = 0.99$, $p < 0.05$), epicatechin ($r = 0.64$ $p < 0.05$) caffeic acid ($r = 0.77$ $p < 0.05$), p-coumaric acid ($r = 0.94$, $p < 0.05$) and ferulic acid ($r = 0.94$, $p < 0.05$) showed a strong positive correlation with the α-glucosidase inhibitory activity. A significant positive correlation was established between FRAP activity and α-glucosidase ($r = 0.88$, $p < 0.05$). Phenolic compounds inhibited the intestinal α-glucosidase activity, and are regarded as a mechanism to exert antidiabetic effects [46]. α-glucosidase facilitates glucose

absorption in the intestines; thus, inhibiting this enzyme could help to reduce the glucose absorption rate and alleviate postprandial hyperglycaemic condition [47].

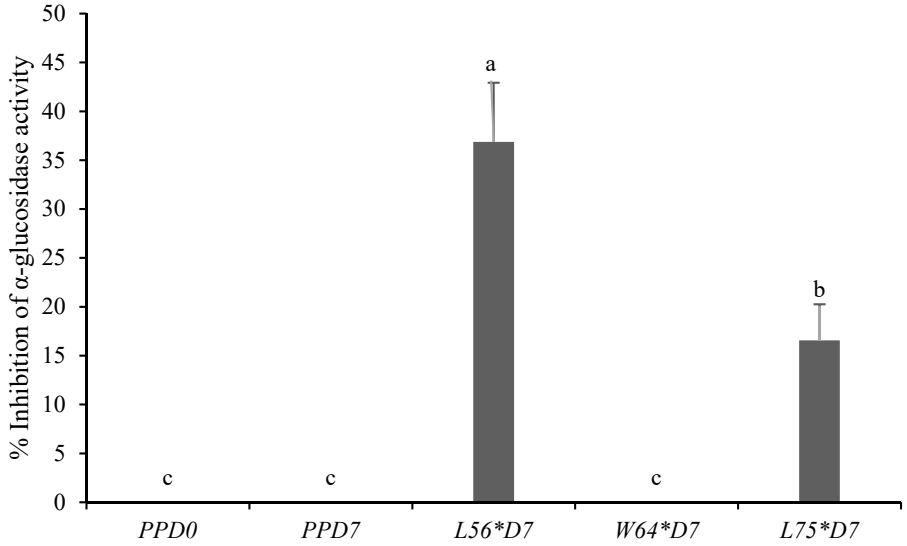

Figure 3. Inhibition of α-glucosidase activity of $\frac{1}{2}$ diluted papaya puree fermented with different LAB strains. Bars with the same letter are not significantly different at $p < 0.05$.

Data are presented as the mean and standard deviation. Bars with different letters indicate significant differences at $p \leq 0.05$. *PPD0*: papaya puree at 0 days of storage; *PPD7*: non-fermented puree stored at 4 °C for 7 days; *L56*D7*: papaya puree fermented with *Leu. pseudomesenteroides* 56 stored at 4 °C for 7 days; *W64*D7*: papaya puree fermented with *W. cibaria* 64 stored at 4 °C for 7 d; *L75*D7*: papaya puree fermented with *Lpb. plantarum* 75 and stored at 4 °C for 7 d.

3.7. In Vitro-Simulated Gastrointestinal (GI) Digestion and Antioxidant Power of Fermented and Non-Fermented Papaya Puree

In order for consumers to be able to utilise the phenolic compounds in food, the increase in the bioaccessibility of polyphenols is important [48]. Therefore, the effect of digestion on the phenolic components, percent recovery and bioaccessibility of fermented and non-fermented purees at the gastric, intestinal and dialysis phase is presented in Tables 4 and 5, respectively.

At the gastric phase, the total phenol content was significantly higher (502.4 mg/100 g DW) in stored *L75*D7* papaya puree. In general, gastric, intestinal and dialysable fractions of non-fermented and fermented puree showed significantly ($p < 0.05$) higher concentrations of phenolic content compared to the respective undigested sample (before fermentation). Therefore, the observed differences could relate to the interaction and interference of the food matrix and interactions with other dietary components, such as fibre, proteins, pH and the enzyme pancreatin. This observation could be due to the hydrolysis of bound phenolic compounds from carbohydrates and proteins from the food matrix facilitated by enzymatic action and low pH [49]. The decrease in pH during fermentation could have increased their stability and extractability [50].

Conversely, the observed differences in total phenol content in the gastric fraction could be due to the difference in the survival or cell population of the LAB strains in the gastrointestinal phase, which is responsible for the higher metabolism and biotransformation of most phenolic compounds [8]. However, the cell population of the LAB strains in the gastrointestinal phase were not quantified in this study. The reduction in the phenolic content in the intestinal fraction was related to the pH changes from acidic to alkaline pH [51]. Furthermore, the molecular arrangement of the different bioactive molecules or interaction effects between the bioactive compounds and other dietary compounds could have affected the total phenolic content in the dialysed fraction [9].

Table 4. Influence of fermentation on simulated *in vitro* gastrointestinal digestion of different phenolic compounds in papaya puree (mg/kg).

Phenolic compounds in papaya puree (mg/kg)																
	PPD7				*L56*D7*				*W64*D7*				*L75*D7*			
Compounds	**BD**	**GP**	**IP**	**DP**	**BD**	**GP**	**IP**	**DP**	**BD**	**GP**	**IP**	**DP**	**BD**	**GP**	**IP**	**DP**
Total phenol	303.9 ± 1.3 ^{g}	407.4 ± 1.7 ^{de}	396.3 ± 2.3 ^{e}	135.4 ± 0.3 ^{i}	294.7 ± 0.1 ^{g}	481.3 ± 0.4 ^{b}	411.3 ± 0.1 ^{d}	400.1 ± 1.1 ^{e}	380.0 ± 0.3 ^{f}	468.2 ± 0.1 ^{c}	367.4 ± 1.3 ^{f}	273.4 ± 0.5 ^{h}	395.4 ± 0.4 ^{e}	502.4 ± 0.4 ^{a}	372.0 ± 2.4 ^{f}	371.3 ± 0.6 ^{f}
FRAP (µmol TEAC/100 g FW)	1.2 ± 0.1 ^{ij}	3.7 ± 0.1 ^{cd}	3.5 ± 1.1 ^{d}	1.1 ± 1.0 ^{j}	2.0 ± 0.3 ^{h}	2.9 ± 0.2 ^{e}	2.5 ± 0.7 ^{f}	1.4 ± 0.0 ^{i}	2.7 ± 0.5 ^{ef}	3.9 ± 1.6 ^{bc}	4.0 ± 0.1 ^{b}	2.0 ± 0.3 ^{h}	2.8 ± 0.2 ^{e}	4.7 ± 1.0 ^{a}	3.7 ± 1.2 ^{c}	2.3 ± 0.1 ^{g}
Gallic acid	6.7± 1.5 ^{f}	6.1 ± 0.2 ^{f}	185.0 ± 1.0 ^{a}	73.7 ± 2.6 ^{d}	5.6 ± 0.1 ^{f}	140.2 ± 2.1 ^{b}	150.9 ± 13.5 ^{b}	47.6 ± 0.1 ^{e}	2.9 ± 0.2 ^{f}	89.2 ± 1.2 ^{d}	126.8 ± 0.6 ^{c}	42.9 ± 6.0 ^{e}	6.4 ± 0.5 ^{f}	57.5 ± 0.4 ^{e}	139.6 ± 0.2 ^{bc}	43.2 ± 1.3 ^{e}
Gallocatechin gallate	564.2 ± 1.8 ^{a}	534.6 ± 7.2 ^{b}	264.3 ± 4.4 ^{f}	32.9 ± 7.6 ^{ij}	218.9 ± 2.4 ^{f}	216.3 ± 1.1 ^{f}	100.4 ± 0.7 ^{h}	16.6 ± 1.3 ^{j}	462.5 ± 9.8 ^{c}	447.3 ± 7.4 ^{c}	158.6 ± 2.3 ^{g}	33.2 ± 1.1 ^{ij}	330.4 ± 1.9 ^{d}	311.7 ± 22.2 ^{e}	111.4 ± 9.5 ^{h}	47.8 ± 4.4 ^{i}
Protocatechuic acid	19.3± 0.6 ^{f}	18.5 ± 0.4 ^{fg}	15.4 ± 0.5 ^{fg}	8.9 ± 0.2 ^{g}	44.4 ± 2.0 ^{c}	32.1 ± 0.4 ^{de}	39.8 ± 1.8 ^{cd}	15.1 ± 0.1 ^{fg}	19.3 ± 0.8 ^{g}	39.4 ± 0.7 ^{cd}	66.1 ± 6.0 ^{a}	24.7 ± 0.6 ^{e}	17.4 ± 0.1 ^{fg}	29.3 ± 2.7 ^{e}	56.6 ± 0.3 ^{b}	18.4 ± 0.2 ^{fg}
Catechin	14.7 ± 1.5 ^{h}	13.4 ± 1.1 ^{h}	26.6 ± 3.5 ^{gh}	5.5 ± 0.2 ^{h}	66.1 ± 2.7 ^{de}	139.2 ± 5.5 ^{c}	211.4 ± 9.8 ^{b}	39.5 ± 1.2 ^{fg}	58.2 ± 0.7 ^{ef}	84.2 ± 6.4 ^{d}	137.4 ± 7.9 ^{c}	41.1 ± 2.3 ^{fg}	51.7 ± 1.3 ^{ef}	121.9 ± 5.4 ^{c}	277.5 ± 6.6 ^{a}	68.3 ± 2.7 ^{de}
Chlorogenic acid	17.2± 0.4 ^{b}	22.1 ±0.2 ^{a}	8.0 ± 0.1 ^{c}	2.2 ± 0.1 ^{e}	17.7 ± 0.2 ^{b}	3.5 ± 1.4 ^{e}	7.6 ± 0.1 ^{c}	0.7 ± 0.2 ^{e}	1.3 ± 0.8 ^{ef}	5.9 ± 2.2 ^{d}	10.0 ± 3.3 ^{c}	4.8 ± 0.4 ^{de}	1.7 ± 0.2 ^{e}	5.6 ± 1.0 ^{d}	10.0 ± 0.1 ^{c}	4.2 ± 0.2 ^{de}
Vanillic acid	4.5± 0.8 ^{c}	4.9 ± 0.5 ^{c}	2.5 ± 0.1 ^{cd}	1.1 ± 0.3 ^{d}	4.5 ± 0.4 ^{c}	4.1 ± 0.8 ^{c}	4.8 ± 0.2 ^{c}	2.1 ± 0.1 ^{cd}	2.5 ± 0.5 ^{cd}	5.4 ± 2.0 ^{bc}	7.5 ± 0.3 ^{bc}	2.5 ± 0.1 ^{cd}	2.5 ± 1.1 ^{cd}	9.7 ± 0.9 ^{ab}	12.7 ± 0.2 ^{a}	2.5 ± 0.3 ^{cd}
Syringic acid	4.9± 0.1 ^{e}	5.1 ± 1.2 ^{e}	17.3 ± 0.4 ^{cd}	8.2 ± 1.0 ^{e}	7.2 ± 0.4 ^{e}	7.9 ± 1.6 ^{e}	20.3 ± 1.5 ^{c}	13.5 ± 0.8 ^{d}	2.5 ± 0.1 ^{ef}	3.7 ± 0.6 ^{ef}	19.3 ± 0.9 ^{cd}	3.4 ± 0.1 ^{ef}	4.8 ± 1.2 ^{ef}	35.2 ± 1.4 ^{a}	29.3 ± 0.9 ^{b}	1.2 ± 1.2 ^{f}
Ellagic acid	5.4± 1.1 ^{d}	5.6 ± 0.3 ^{cd}	11.2 ± 0.7 ^{c}	8.1 ± 0.7 ^{cd}	6.2 ± 1.5 ^{cd}	4.3 ± 2.0 ^{d}	13.3 ± 0.9 ^{bc}	7.1 ± 0.1 ^{cd}	3.1 ± 0.2 ^{d}	4.8 ± 1.3 ^{d}	12.2 ± 0.4 ^{bc}	6.2 ± 0.4 ^{cd}	12.4 ± 0.2 ^{bc}	19.3 ± 2.4 ^{b}	29.1 ± 0.7 ^{a}	2.8 ± 0.6 ^{d}
Quercetin	103.8± 16.3 ^{a}	87.5 ± 2.3 ^{b}	62.8 ± 0.9 ^{c}	38.1 ± 0.8 ^{f}	38.1 ± 1.5 ^{f}	31.7 ± 1.7 ^{fg}	27.4 ± 2.9 ^{gh}	15.9 ± 0.4 ^{i}	52.8 ± 5.0 ^{e}	58.1 ± 2.4 ^{de}	63.2 ± 3.1 ^{cd}	25.9 ± 0.8 ^{g}	57.7 ± 2.3 ^{de}	68.9 ± 2.1 ^{bc}	73.5 ± 3.1 ^{b}	18.8 ± 2.3 ^{hi}
p-Coumaric acid	23.7± 2.5 ^{ef}	21.6 ± 0.7 ^{ef}	113.8 ± 10.0 ^{a}	54.4 ± 0.9 ^{c}	37.4 ± 3.2 ^{d}	32.5 ± 0.6 ^{de}	29.0 ± 1.6 ^{de}	16.7 ± 0.5 ^{f}	17.3 ± 0.8 ^{f}	74.2 ± 1.6 ^{c}	87.2 ± 0.7 ^{b}	24.0 ± 5.8 ^{ef}	25.4 ± 0.8 ^{ef}	58.1± 3.7 ^{c}	84.4 ± 2.7 ^{b}	17.5 ± 0.9 ^{f}
Ferulic acid	12.6± 0.1 ^{f}	13.4 ± 0.9 ^{ef}	15.8 ± 0.2 ^{e}	6.0 ± 0.1 ^{g}	20.5 ± 0.1 ^{bc}	18.9 ± 1.1 ^{cd}	17.2 ± 0.5 ^{de}	11.5 ± 0.3 ^{f}	13.5 ± 0.^{ef}	15.0 ± 0.8 ^{e}	18.9 ± 0.1 ^{cd}	7.0 ± 0.7 ^{g}	14.3 ± 0.3 ^{e}	19.2 ± 1.1 ^{c}	27.4 ± 0.4 ^{a}	12.1 ± 0.2 ^{f}

Values are mean ± standard deviation, and means followed by a different letter within the row are significantly different ($p \leq 0.05$). BD: before digestion; GP: gastric phase; IP: intestinal phase; DP: dialysis phase; DW: dry weight; *PPD7*: non-fermented papaya puree stored at 4 °C for 7 d; *L56*D7*: papaya puree fermented with *Leuconostoc pseudomesenteroides* 56 stored at 4 °C for 7 d; *W64*D7*: papaya puree fermented with *Weissella cibaria* 64 stored at 4 °C for 7 d; *L75*D7*: papaya puree fermented with *Lpb. plantarum* 75 and stored at 4 °C for 7 d.

Table 5. Recovery and bioaccessibility (%) of different phenolic compounds in fermented and non-fermented papaya puree.

	PPD7			***L56*D7***			***W64*D7***			***L75*D7***		
	Recovery%		**Bioaccessibility%**	**Recovery%**		**Bioaccessibility%**	**Recovery%**		**Bioaccessibility%**	**Recovery%**		**Bioaccessibility%**
Phenolic Compounds	**GP**	**IP**	**DP**	**GP**	**IP**	**DP**	**GP**	**IP**	**DP**	**GP**	**IP**	**DP**
Gallic acid	91.0 ± 3.0 ^{i}	2761.2 ± 5.7 ^{c}	1100.0 ± 6.2 ^{f}	2503.6 ± 6.0 ^{c}	2694.6 ± 3.9 ^{c}	850.0 ± 3.0 ^{g}	3075.9 ± 1.8 ^{b}	4372.4 ± 5.9 ^{a}	1479.3 ± 2.8 ^{e}	898.4 ± 2.9 ^{g}	2181.3 ± 3.3 ^{d}	675.0 ± 1.5 ^{h}
Gallocatechin gallate	94.8 ± 1.0 ^{ab}	46.8 ± 0.8 ^{b}	5.8 ± 0.4 ^{f}	98.8 ± 1.9 ^{a}	45.9 ± 2.0 ^{b}	7.6 ± 0.4 ^{e}	96.7 ± 2.5 ^{a}	34.3 ± 1.9 ^{c}	7.2 ± 0.5 ^{e}	94.3 ± 1.7 ^{ab}	33.7 ± 2.4 ^{c}	14.5 ± 1.7 ^{d}
Protocatechuic acid	95.9 ± 2.1 ^{e}	79.8 ± 3.0 ^{f}	46.1 ± 2.2 ^{h}	72.3 ± 2.4 ^{g}	89.6 ± 3.1 ^{e}	34.0 ± 0.8 ^{i}	204.1 ± 3.1 ^{b}	342.5 ± 2.8 ^{a}	128.0 ± 1.9 ^{cd}	168.4 ± 2.9 ^{c}	325.3 ± 2.0 ^{ab}	105.7 ± 3.3 ^{d}
Catechin	91.2 ± 1.7 ^{g}	181.0 ± 2.0 ^{d}	37.4 ± 0.5 ^{j}	210.6 ± 1.8 ^{cd}	319.8 ± 3.4 ^{b}	59.8 ± 3.7 ^{i}	144.7 ± 2.9 ^{e}	236.1 ± 2.2 ^{c}	70.6 ± 2.9 ^{h}	235.8 ± 3.6 ^{c}	536.8 ± 1.8 ^{a}	132.1 ± 2.0 ^{f}
Epicatechin	87.1 ± 2.1 ^{e}	164.5 ± 2.7 ^{b}	59.7 ± 0.6 ^{f}	86.4 ± 1.0 ^{e}	114.8 ± 2.1 ^{c}	32.5 ± 0.8 ^{h}	109.1 ± 1.5 ^{d}	137.7 ± 1.6 ^{bc}	40.3 ± 0.8 ^{g}	140.7 ± 1.9 ^{bc}	207.4 ± 2.2 ^{a}	79.6 ± 1.6 ^{e}
Caffeic acid	89.1 ± 0.8 ^{b}	100.0 ± 2.9 ^{a}	28.3 ± 1.2 ^{e}	37.1 ± 1.1 ^{d}	43.8 ± 0.9 ^{c}	12.4 ± 0.5 ^{f}	97.8 ± 2.0 ^{ab}	100.0 ± 2.3 ^{a}	37.0 ± 4.0 ^{d}	93.5 ± 1.5 ^{b}	100.0 ± 2.0 ^{a}	106.5 ± 3.0 ^{a}
Chlorogenic acid	128.5 ± 1.1 ^{e}	46.5 ± 0.8 ^{f}	12.8 ± 0.1 ^{h}	19.8 ± 0.9 ^{g}	42.9 ± 0.8 ^{f}	4.0 ± 0.1 ^{i}	453.8 ± 2.1 ^{bc}	769.2 ± 1.8 ^{a}	369.2 ± 3.1 ^{c}	329.4 ± 2.4 ^{c}	588.2 ± 1.5 ^{b}	247.1 ± 0.7 ^{d}
Vanillic acid	108.9 ± 3.3 ^{d}	55.6 ± 2.0 ^{f}	24.4 ± 0.7 ^{h}	91.1 ± 2.9 ^{e}	106.7 ± 1.0 ^{d}	46.7 ± 2.2 ^{g}	216.0 ± 0.9 ^{c}	300.0 ± 3.4 ^{bc}	100.0 ± 0.9 ^{d}	388.0 ± 2.0 ^{b}	508.0 ± 1.0 ^{a}	100.0 ± 0.4 ^{d}
Syringic acid	104.1 ± 2.9 ^{g}	353.1 ± 2.7 ^{c}	167.3 ± 2.0 ^{e}	109.7 ± 3.1 ^{g}	281.9 ± 1.2 ^{d}	187.5 ± 4.5 ^{de}	148.0 ± 3.4 ^{f}	772.0 ± 6.0 ^{a}	136.0 ± 1.8 ^{f}	733.3 ± 3.0 ^{ab}	610.4 ± 1.9 ^{b}	25.0 ± 0.5 ^{h}
Ellagic acid	103.7 ± 2.9 ^{f}	207.4 ± 0.8 ^{c}	150.0 ± 1.9 ^{e}	69.4 ± 1.7 ^{g}	214.5 ± 1.1 ^{c}	114.5 ± 0.7 ^{f}	154.8 ± 1.2 ^{e}	393.5 ± 2.0 ^{a}	200.0 ± 1.9 ^{d}	155.6 ± 2.1 ^{e}	234.7 ± 2.8 ^{b}	22.6 ± 0.9 ^{h}
Quercetin	84.3 ± 2.0 ^{c}	60.5 ± 1.1 ^{d}	36.7 ± 0.6 ^{f}	83.2 ± 1.4 ^{c}	71.9 ± 2.2 ^{cd}	41.7 ± 0.7 ^{e}	110.0 ± 2.8 ^{b}	119.7 ± 1.6 ^{ab}	49.1 ± 0.1 ^{e}	119.4 ± 2.0 ^{ab}	127.4 ± 1.9 ^{a}	32.6 ± 2.2 ^{f}
p-Coumaric acid	91.1 ± 2.0 ^{f}	480.2 ± 2.7 ^{ab}	229.5 ± 3.5 ^{d}	86.9 ± 2.5 ^{f}	77.5 ± 1.8 ^{g}	44.7 ± 2.6 ^{h}	428.9 ± 4.1 ^{b}	504.0 ± 2.1 ^{a}	138.7 ± 1.9 ^{e}	228.7 ± 2.8 ^{d}	332.3 ± 3.3 ^{c}	68.9 ± 1.7 ^{g}
Ferulic acid	106.3 ± 1.3 ^{d}	125.4 ± 0.9 ^{c}	47.6 ± 1.2 ^{h}	92.2 ± 2.5 ^{e}	83.9 ± 0.9 ^{e}	56.1 ± 1.3 ^{f}	111.1 ± 0.9 ^{d}	140.0 ± 2.8 ^{b}	51.9 ± 3.6 ^{g}	134.3 ± 1.9 ^{b}	191.6 ± 2.1 ^{a}	84.6 ± 3.5 ^{e}

Values are mean ± standard deviation, and means followed by a different letter within the row are significantly different ($p \leq 0.05$). BD: before digestion; GP: gastric phase; IP: intestinal phase; DP: dialysis phase; DW: dry weight; *PPD7*: non-fermented papaya puree stored at 4 °C for 7 d; *L56*D7*: papaya puree fermented with *Leu. pseudomesenteroides* 56 stored at 4 °C for 7 d; *W64*D7*: papaya puree fermented with *W. cibaria* 64 stored at 4 °C for 7 d; *L75*D7*: papaya puree fermented with *Lpb. plantarum* 75 and stored at 4 °C for 7 d.

The undigested non-fermented puree stored for 7 days at 4 °C contained the highest concentration of gallocatechin gallate (564.2 mg/kg), and its concentration significantly ($p < 0.05$) decreased with fermentation, while *W64*D7*-fermented puree had the highest concentration (462.5 mg/kg). The gallocatechin gallate concentration at the intestinal phase of all fermented and non-fermented purees showed a substantial reduction, varying from 264.3 to 100.4 mg/kg compared to the gastric fraction and the undigested purees. The % recovery of gallocatechin gallate in the intestinal fraction was 46.8%, 45.9%, 34.3% and 33.7% in *PPD7*, *L56*D7*, *W64*D7* and *L75*D7*, respectively, while 14.5% was recovered in the *L75*D7* dialysed fraction. The intestinal fractions of non-fermented and fermented purees showed a substantial increase in gallic acid concentration compared to the undigested and gastric fractions. The amount of gallic acid bioaccessible in the dialysable fraction of the non-fermented puree was 1100.0% compared to its undigested sample. The % recovery of gallic acid in the dialysed fractions of W*64*D7, L56*D7* and *L75*D7* was 1479.3%, 850% and 675.0%, respectively.

Krook and Hagerman [51] reported the stability of epigallocatechin-O-gallate at pH < 1.5 and 5–6, and its degradation at pH higher than seven produced gallic acid. Therefore, it can be hypothesised that the gallocatechin gallates could be stable at pH 2, and at pH 7 due to its instability, and could undergo decomposition that produces gallic acid, especially at the intestinal phase. Furthermore, Liu et al. [52] also showed that the increase in gallic acid due to alkaline hydrolysis could had released the bound phenolic acids, thus increasing their bioavailability. On the contrary, Tagliazucchi et al. [52] and Jara-Palacios et al. [53] reported the degradation of gallic acid at the intestinal phase. However, the *L56*D7* and *L75*D7* could have partially metabolised, thereby reducing the % recovery of gallic acid in the intestinal fraction of the fermented purees.

Similar to the report of Jara-Palacios et al. [53] on the higher concentration of protocatechuic acid in the intestinal digests of Zalema grapes (*Vitis vinifera* sp.) pomace. Lui et al. [54] also showed increased extraction of phenolic acids under mild alkaline conditions.

In this study, samples *W64*D7* and *L75*D7* had higher protocatechuic acid concentrations than the undigested samples (PPD7). Additionally, the higher gallic acid concentration at the intestinal phase indicates that the gallic acid did not undergo a dehydroxylation process for the production of protocatechuic acid [9]. There was a significantly higher concentration of protocatechuic acid in the *W64*D7* and *L75*D7* at the gastric phase ($p < 0.05$) than the *L56*D7.* The % recovery of protocatechuic acid was significantly higher in the *W64*D7*- (342.5%) and *L75*D7* (325.3%)-fermented purees at the intestinal phase compared to the *L56*D7* and undigested purees ($p \leq 0.05$). W*64*D7* and *L75*D7* had a significantly higher % recovery of protocatechuic acid at the dialysis phase compared to the other samples. It is possible that the protocatechuic acid could have been partially metabolised by *L56*D7* during gastric digestion, since it was reduced from 44.4 to 32.1 mg/kg.

Catechin concentration increased in the gastric fraction of the fermented puree. This could be due to the lower pH of the fermented purees and the lower gastric pH, which resulted in stable catechin molecules [52]. At the same time, catechin concentration increased significantly in the intestinal fractions of fermented purees. The intestinal fraction of *L75*D7* showed a significantly higher amount (277.5 mg/kg) of catechins with 536.8% recovery. Moreover, the dialysable fraction of *L75*D7* showed the highest bioaccessible catechin (132.1%) compared to the undigested, digested fermented and non-fermented purees. The stability of catechins has been correlated with the pH and are reported to be stable in acidic conditions, and unstable at pH greater than or near neutral [55]. The observed increase in catechin in the intestinal fraction in this study could be due to the spontaneous degradation of gallocatechins at alkaline pH [9].

The percentage recovery of ellagic acid in the gastric fraction of L*56*D7* (69.4%) was lower when compared to its undigested sample (103.7%), *W64*D7* (154.8%) and *L75*D7* (155.6%). This suggests the possible utilisation of ellagic acid by *L56*D7.* Conversely, the % of ellagic acid was significantly ($p < 0.05$) increased in the intestinal fractions of the

non-fermented and fermented puree compared to their undigested samples. The observed increase in ellagic acid concentration could be due to the hydrolysis of ellagitannins from the food matrix to ellagic acid due to the mild alkaline pH (7.5) at the intestinal phase [56]. The highest % bioaccessibility of ellagic acid was in the *W64*D7* (200.0%) dialysed fraction and was significantly different to the dialysed digest of the other purees. The observed lower % bioaccessibility (22.6%) of ellagic acid in the dialysed fraction of *L75*D7* digest could be due to the possible utilisation of ellagic acid during fermentation caused by *Lpb. plantarum*. *Lactobacillus* spp. reportedly has the ability to utilise ellagic acid and glycosyl ellagic acid during metabolism [57].

Furthermore, the percentage recovery of chlorogenic acid and syringic acid was significantly higher in the intestinal fraction of *W64*D7* (769.2%; 772.0%), with the highest % bioaccessible amount of 369.2% chlorogenic acid and 136.0% syringic acid at the dialysis phase. Moreover, fermentation increased the % recovery of vanillic acid in the intestinal fractions and was highest in *L75*D7* (508.0%). The highest percentage bioaccessible content of 100% was obtained in *W64*D7* and *L75*D7* in the dialysable fractions of fermented purees. The increase in ellagic, chlorogenic, syringic and vanillic acid concentrations after intestinal digestion could be due to their release from their bound form in the food matrix due to enzymatic digestion [9]. The decrease in chlorogenic acid, ellagic acid and ferulic acid in *L56*D7* at the gastric phase could be due to the partial use of these compounds during metabolism.

LAB fermentation caused an increase in the quercetin content of fermented purees compared to the non-fermented purees. The percentage recovery of quercetin content was higher in the *W64*D7* (119.7%; 110.0%) and *L75*D7* (127.4%; 119.4%) compared to *L56*D7* (71.9%; 83.2%) and *PPD7* (60.5%; 84.3%) at both intestinal and gastric phases, respectively. *L75*D7* had the highest % recovery of quercetin at the intestinal phase and was not significantly different to *W64*D7* ($p > 0.05$). Likewise, the dialysable fractions of *L56*D7* (41.7%) and *W64*D7*, despite being high, were not significantly different to one another ($p > 0.05$). A similar non-significant change in the quercetin content was reported during the gastric and intestinal phase digestion of onions [58]. These results, therefore, suggest that the pH change during fermentation and during the gastric, intestinal phases and the action of digestive enzymes (pancreatin), could have participated in the release of quercetin from the food matrix [58]. The degree of metabolization by different LAB strains used in this study varied, as reflected in the p-coumaric acid concentration found in the fermented samples. The observed non-significant reduction in p-coumaric acid in the undigested *L56*D7* puree could be due to the decarboxylation of ferulic acid [59].

The intestinal and dialysed fractions of *W64*D7* had the highest p-coumaric acid % recovery (504.0%) and % bioaccessibility (138.7%) and were significantly higher than those of other purees. A possible reason for the higher recovery of ferulic acid at the dialysis phase, with respect to the undigested samples, could be due to an interference from the food matrix and the reduced esterification of ferulic acid with sugar moieties after digestion [60]. Similarly, a higher % recovery of ferulic acid was observed in *W64*D7* and *L75*D7* intestinal fractions than their respective gastric fractions. *L75*D7* had the highest % bioaccessible ferulic acid at the dialysable fraction. The observed decrease in the % recovery of different phenolic constituents at the intestinal phase in *L56*D7* suggests a partial metabolism of the phenolic compound. Valero-Cases et al. [8] previously reported the impact of LAB fermentation on the *in vitro* digestion and biotransformation of phenolic compounds in fermented pomegranate juices. Therefore, the relationship among the concentrations of phenolic compounds in fermented puree could be correlated with the increased LAB survival in *L75*D7* and *L56*D7* during and after fermentation.

3.8. Effect of Fermentation and In Vitro Digestion on the Antioxidant Capacity of Papaya Puree

The types of transformations, such as epimerisation, degradation, oxidation and hydrolysis, during the fermentation and gastrointestinal digestion can affect the phenolic content and its structure [8]. Fermentation with LAB strains increased the antioxidant

capacity (FRAP values) of papaya purees, with the exception of *L56*D7*. During gastric digestion, the FRAP values of *PPD7*, *W65*D7* and *L75*D7* significantly ($p \leq 0.05$) increased, compared to the undigested samples. The highest FRAP antioxidant power was obtained in the gastric fraction of *L75*D7* (4.7 µmol TEAC/100 g FW), while a significant ($p \leq 0.05$) decrease in FRAP was observed in the intestinal fractions of *L56*D7* and *L75*D7*, but not *W64*D7*, when compared to the gastric fractions. The FRAP values in the dialysable fractions range was lowest in *PPD7* (1.1 µmol TEAC/100 g FW) and highest in *L75*D7* (2.3 µmol TEAC/100 g FW). The findings in this study confirm that fermentation with *Lpb. plantarum 75* and *W. cibaria 64* increases the FRAP activity due to the contribution of the free soluble antioxidants. The FRAP activity and total phenol content are positively correlated [46]. The metal chelating properties of phenolic constituents contributed to the antioxidant activity of *W64*D7* and *L75*D7*, which can be justified by the increase in most phenolic constituents compared to in the *L56*D7* and non-fermented papaya puree.

4. Conclusions

The results presented in this study showed that the fermentation of papaya puree by *W. cibaria 64* and *Lpb. plantarum 75* improves antioxidant capacity (FRAP activity) due to the increase in phenolic constituents compared to the *Leu. pseudomesenteroides 56* and non-fermented papaya puree. However, the viability of all LAB strains used in this study after *in vitro* digestion requires investigation. This study provided important information on the estimation based on the percentage recovery of different phenolic constituents that are available for *in vivo* absorption after the consumption of LAB-fermented papaya puree. However, further investigations are necessary in the survival of LABs; antioxidant activity after in vitro digestion and the bioaccessibility of phenolic constituents after digestion could be investigated using Caco-2 cellular models to confirm the uptake of phenolic and carotenoid components. Based on the phenolics profiles, antioxidants, LAB survival and quality parameters of the purees, the study recommends that local food manufacturers in Reunion Island use *Lpb. plantarum 75* for the fermentation of papaya purees for optimum nutrient bioaccessiblity and functional benefits from locally produced papaya.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10050962/s1, Table S1: The identification of different phenolic compounds.

Author Contributions: F.M.M.—performed the analysis and wrote the first draft; S.A.A.—executed the data validation, visualisation and revised the fermentation part of the article; V.E.M.—executed the data validation of the HPLC analysis and data presentation; C.G.—guidance and methodology for *in vitro* intestinal investigation; F.R.—was responsible for the supervision of the fermentation analysis; R.M.S.— edited the manuscript; D.S.—conceptualisation, project administration, data validation and final editing. All authors have read and agreed to the published version of the manuscript.

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ARTICLES FOR FACULTY MEMBERS

EFFECT OF LACTIC ACID BACTERIA AS BIO-PRESERVATION AGAINST SPOILAGE FUNGI FROM PAPAYA FRUIT

Lactic acid bacteria: Beyond fermentation to bio-protection against fungal spoilage and mycotoxins in food systems / Rahman, M. S., Soltani, S., LaPointe, G., Karboune, S., & Fliss, I.

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Lactic acid bacteria: beyond fermentation to bio-protection against fungal spoilage and mycotoxins in food systems

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Recent outbreaks of foodborne diseases have highlighted the challenges of maintaining food safety, emphasizing the need for effective strategies to control pathogens and spoilage organisms. Toxins produced by indigenous fungi pose serious economic issues and undermine food security. Mycotoxin spoilage is a ubiquitous hazard that affects all food commodities; however, bakery products, dairy, fruits, vegetables, and meat are particularly vulnerable. The quality of food is perceived through senses such as taste, aroma, and texture. These sensory attributes significantly impact the overall sensation of the product and determine whether it will be accepted or rejected by consumers. Spoilage not only reduces consumer satisfaction but also drastically shortens the shelf life of food. This review highlights the ability of Lactic Acid Bacteria (LABs) to produce diverse antimicrobials, emphasizing antifungal metabolites as effective tools for enhancing food preservation and extending shelf life. As consumer demand for 'clean label' solutions increases, these natural antimicrobials promise safe and effective alternatives for enhancing food safety, reducing fungal spoilage, and extending shelf life of various perishable food commodities and reducing economic losses.

KEYWORDS

antimicrobial, lactic acid bacteria (LAB), bio-preservation, shelf-life, fungal spoilage, mycotoxins

Introduction

Fungi comprise more than 1 million species and, unlike plants, lack chlorophyll, relying instead on external sources of organic matter. They thrive in damp, dark environments (Moore et al., 2020). Mold, a common fungal form, frequently contaminates food due to their widespread distribution in nature and adaptability to various conditions. Contamination often occurs in food processing facilities via raw materials, surfaces, and equipment. In unhygienic environments, fungal spores can travel through air and adhere to clothing or footwear, potentially introducing pollutants into production areas. Fungal contamination negatively impacts the quality, safety, and longevity of feed and food commodities, causing significant economic losses. Studies indicate that fungal spoilage accounts for approximately 5–10% of global food production losses and 50% of fruit and vegetable waste in tropical regions (Mastanjević et al., 2022). Australia alone incurs around $10 million annually in food losses attributed to fungal spoilage (Cheong et al., 2014), while bread spoilage costs in Western

Europe exceed €200 million annually (Abdelhameed and Khalifa, 2024). Crop contamination by fungi such as *Aspergillus* and *Fusarium* also results in agricultural losses of up to $60 billion per year.

In addition to spoilage, some fungi can pose serious risks to food and feed safety through the production of mycotoxins, which are harmful secondary metabolites produced by certain filamentous fungi, particularly from the genera *Aspergillus*, *Penicillium*, and *Fusarium*. Major mycotoxins include ochratoxins, fumonisins, and aflatoxins, which have been linked to liver cancer, nephropathy, immunosuppression, and growth impairment in both humans and animals (Bryden, 2007; Milićević et al., 2016). Aflatoxins, which contaminate food consumed by an estimated 4.5 billion individuals in less economically developed nations, can cause acute poisoning resulting in death in approximately 40% of cases, as documented in Kenya (CDC, 2004). Aflatoxin B1 (AFB1), produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*, is the most prevalent form. Other types such as G1 and M2 are found in grains and dairy products, respectively (Min et al., 2021). The World Health Organization (WHO) has classified aflatoxins as Group 1 human carcinogenic (Kensler et al., 2011).

Beyond aflatoxins, other foodborne molds also produce toxic secondary metabolites that persist even after processing (Bullerman and Bianchini, 2007), posing chronic risks to immunocompromised individuals (Milićević et al., 2010). The prevalence of fungal contamination and mycotoxin production in food and feed systems remains a global concern, with some estimates suggesting that up to 25% of the world's food supply is affected, raising significant alarms among researchers, manufacturers, and regulatory bodies (Topping and Clifton, 2001). Notably, species such as *Candida* spp., *Fusarium* spp., and *Aspergillus* spp. have been identified as opportunistic pathogens capable of causing systemic infections in immunocompromised hosts.

Despite their detrimental effects, certain fungi and yeasts contribute positively to the food industry. They play important roles in traditional fermentation processes, improving organoleptic properties such as flavor, aroma, and texture, as well as enhancing nutritional value through the production of organic acids, enzymes, and other bioactive compounds (Pouris et al., 2024). Genera such as *Pichia, Geotrichum, Candida, Zygosaccharomyces, Kluyveromyces, Torulaspora, and Saccharomyces*, along with molds such as *Aspergillus, Penicillium, Geotrichum, Phoma, Mucor*, and *Rhizopus*, are frequently employed as stater cultures in the fermentation of cereals, meats, and milk (Rai and Jeyaram, 2017; Wei et al., 2019). Nonetheless, contamination by acid-tolerant fungi during fermentation remains a persistent risk (Dinakarkumar et al., 2024).

Chemical additives, such as potassium sorbate, sulfur dioxide, and calcium propionate, are commonly used to combat food spoilage by yeasts and molds. However, these additives fail to match the rising demand of consumers for "clean label" products made with natural ingredients (Leyva Salas et al., 2017). Alternative methods for detoxifying or removing mycotoxins from feed or food products include physical methods such as the use of ultraviolet light, ionizing radiation, or heat; chemical processes such as acid or alkaline-based methods; and hydrolytic, chlorinating, oxidizing, or reducing agents. However, some fungal strains and their mycotoxins are resistant to these methods, which can negatively impact product quality and increase processing costs (Molina-Hernandez et al., 2025). Therefore, innovative, natural, safe, and cost-effective solutions are required to address fungal contamination in food.

In this context, bio-preservation or the utilization of precisely identified microbes or their respective antimicrobial substances in food, has risen, driven by an expanding consumer preference for more natural preservation methods as opposed to synthetic chemicals (Pouris et al., 2024). Among these, lactic acid bacteria (LAB) have emerged as strong candidates for antifungal biopreservation. For decades, LAB has been utilized in conventional food fermentations and many species have been granted 'Generally Recognized as Safe' (GRAS) and 'Qualified Presumption of Safety' (QPS) certification through the European Food Safety Authority (EFSA) and the American Food and Drug Administration (FDA) (Leuschner et al., 2010). LAB are defined as Gram-positive, non-spore-forming rods or cocci, that ferment carbohydrates to produce lactic acid as their main metabolic end product. LAB are commonly classified into three physiological groups based on their fermentation pathways: obligate homofermentative, which produce lactic acid as the sole end-product from glucose; obligate heterofermentative, which convert glucose into lactic acid, ethanol or acetic acid, and CO_2; and facultative heterofermentative, which primarily produce lactic acid from glucose but can shift to heterofermentative pathways under specific conditions (Mokoena, 2017).

LAB strains have been referred to as "green preservatives" because they could suppress several undesirable microorganisms in food, including fungi, through the production of natural antimicrobial compounds. In the context of food safety, green preservatives refer to naturally derived or minimally processed substances that offer microbial control without the use of synthetic chemical additives, aligning with consumer demand for clean-label and safer food products (Pawlowska et al., 2012). Many LAB strains are known to produce a variety of antifungal substances including alcohols, lactones, aldehydes, diacetyl, organic acids, bioactive antimycotic peptides, hydrogen peroxide, carboxylic acids, fatty acids, and bacteriocins (Crowley et al., 2013). Recently, LAB strains have been used to control fungi in both fermented and non-fermented foods, including cereals, yogurt and fresh products (Aunsbjerg et al., 2015). In addition to inhibiting fungal growth, some LAB strains can bind and neutralize mycotoxins via interactions with their cell wall components.

However, several limitations affect the widespread application of LAB in food systems. Their antifungal activity is highly strain-specific and may vary depending on food matrix composition. Moreover, the stability of bioactive metabolites under processing and storage conditions poses a challenge. Regulatory constraints can also limit the use of novel LAB strains in food products, despite their recognized safety in traditional applications.

Although multiple scholarly reviews have described the antifungal properties of LAB, most focus on their metabolites diversity or general application without deeply exploring LAB-mycotoxin interactions or practical performance in real food matrices (Crowley et al., 2013; Liu A. et al., 2022; Nasrollahzadeh et al., 2022; Shi and Maktabdar, 2022) This review aims to bridge that gap by critically evaluating the mechanisms through which LAB detoxify mycotoxins, exploring LAB-fungi interactions in food systems, and identifying promising LAB strains for future bio-preservation applications in both fermented and non-fermented products.

This narrative review was based on a non-systematic literature search conducted primarily through ScienceDirect, PubMed, Scopus, and Google Scholar. Keywords included "lactic acid bacteria," "mycotoxins," "food spoilage fungi," "bio-preservation," and

"antifungal metabolites." Preference was given to peer-reviewed articles published between 2010 and 2024, with emphasis on studies from the last 5–7 years. Older but widely cited or foundational sources were included when recent data were unavailable.

Fungi in food products

Fungi significantly contribute to enhancing the flavor of various food items because of their metabolic activities and the production of a wide range of aroma molecules, organic acids, and flavor-active compounds. On the other hand, some fungi cause defects that may be visible or invisible, such as undesirable odors and tastes, leading to substantial food waste and financial setbacks (Melini and Melini, 2024). Mitigating fungal spoilage is a significant challenge for industry professionals, and researchers are seeking effective methods to prevent and minimize fungal contamination of many types of food products (Mhlongo et al., 2019).

Climate change is increasingly recognized as a critical factor influencing the prevalence and distribution of mycotoxigenic fungi. Environmental stressors such as elevated temperatures, high humidity, drought followed by rehydration, and rising atmospheric CO_2 levels have been shown to affect fungal growth and mycotoxin production (Chhaya et al., 2022). These factors can alter gene expression related to toxin biosynthesis (Verheecke-Vaessen et al., 2019). For example, aflatoxin production by *A. flavus* influences under specific combinations of high temperature, water activity, and CO_2 concentration (Medina et al., 2014; Medina et al., 2013). Likewise, extreme rainfall increases deoxynivalenol (DON) accumulation in cereals at harvest (Franz et al., 2009), and elevated CO_2 has been associated with increased plant susceptibility to *Fusarium* infection (Vaughan et al., 2016). Such ecological shifts have facilitated the spread of aflatoxigenic species into temperate regions such as Central and Southern Europe (Kos et al., 2020), raising concerns about the adequacy of traditional storage and food safety practices under changing climatic conditions.

Fungi in bakery products

Bread is particularly vulnerable to fungal contamination, as bakery products are perishable due to moisture loss and microbiological decomposition. Fungal spoilage is considered the leading cause of deterioration in these products, often surpassing bacterial spoilage in frequency and severity (Abdelhameed and Khalifa, 2024). Currently, weak organic acids such as calcium propionate are added to protect food matrices. However, in recent years, both legislators and consumers have advocated for the removal of preservatives from food products due to growing concerns about their potential health risks, including allergic reactions, gut microbiota disruption, and links to chronic diseases (Li et al., 2024). This demand has also been driven by the clean-label movement, which favors minimally processed products with recognizable, natural ingredients. Nonetheless, reducing the number of additives used to prevent mold spoilage in bakery items may, in most cases, decrease the shelf life of the product (Garcia et al., 2019a). In response, there is increasing interest in natural preservation strategies, including the use of antimicrobial metabolites produced by lactic acid bacteria (LAB), essential oils, plant extracts, and biopreservative packaging systems that aim to maintain product safety while meeting consumer expectations (Castellano et al., 2017). Molds in bread pose a significant economic issue, leading to 1–5% product losses depending on the season, product type, and processing technique (Kam et al., 2007). In Europe alone, economic losses due to fungal contamination in bread have been estimated at over €200 million per year, while in tropical regions, spoilage-related losses in baked goods may reach up to 11% (Garcia et al., 2019a). Several studies have identified various species involved in the mold spoilage of bread, with those belong to *Eurotium, Aspergillus*, and *Penicillium* being the most common and significant genera. *Cladosporium*, *Mucor*, and *Rhizopus* have also been found in bread products; however, their higher water activity (Aw) requirements, along with sensitivity to factors such as pH, temperature, and oxygen availability, make them less likely to contaminate bakery products under typical storage conditions (Garcia et al., 2021).

In addition to the economic losses associated with bread goods, mycotoxin production poses a potential health risk. The *Eurotium* species typically establishes itself as the primary fungus that infests inadequately stored or dried products. Their growth increases the water activity (Aw), facilitating the proliferation of a number of other species (e.g., *Penicillium* sp. and *Aspergillus* sp.). Given that *Eurotium* sp. does not generate substantial mycotoxin quantities, it is essential to comprehend how *Aspergillus* and *Penicillium* species can flourish and contaminate bread products, as some species are capable of doing so. Aflatoxicosis, caused by the ingestion of aflatoxins primarily produced by *A. flavus*, remains a significant public health concern, with over 500 reported cases and 200 deaths worldwide since 2004 due to contaminated food products, including bakery items (Shabeer et al., 2022). For instance, in the United States, toxigenic *A. flavus* was recovered from three out of the 15 home bakery products inspected, including a toxigenic *Penicillium* strain (species not specified) from wheat flour and bread (Girardin, 1997). Numerous *Penicillium* species, including *Penicillium chrysogenum*, produce mycotoxins (Garcia et al., 2021). Despite baking temperatures reaching 200–250°C, certain mycotoxins such as ochratoxin A and aflatoxins produced by *Aspergillus* and *Penicillium* spp. can persist due to their thermal stability (Garcia et al., 2019b; Kabak, 2009). OTA and aflatoxins, produced by *Aspergillus* and *Penicillium*, can remain active even after baking due to their thermal stability, posing long-term risks including immunosuppression and carcinogenicity (Gupta et al., 2022). The presence of such mycotoxins in flour or via post-processing contamination is a growing concern for consumer safety and regulatory compliance.

Post-baking yeast contamination is also a major contributor to spoilage. Since baking eliminates most microorganisms, contamination occurs during post-baking stages such as cooling, slicing, packaging, and storage (Vermelho et al., 2024). Airborne yeasts may originate from poorly sanitized equipment (e.g., slicers, conveyor belts, racks), packaging materials, or humid storage environments (Ali et al., 2023).

Evidence of superficial yeast growth on products indicates yeast spoilage (cream or white patches). *Hyphopichia burtonii* (formerly *Pichia burtonii*) often referred to as "chalk mold," is a common spoilage yeast, often forming cream or white patches on bread prior to mold development. Its rapid proliferation and resistance to standard storage practices make it particularly problematic.

Preventive strategies focus on minimizing contamination routes. These include high-efficiency particulate air (HEPA) filtration in production areas, improved sanitation of equipment, and reduced manual handling. Modified-atmosphere packaging (MAP), incorporation of antifungal compounds derived from lactic acid bacteria (LAB), and control of relative humidity are effective methods to extend shelf life and suppress yeast growth (Garnier et al., 2017). In British bread, although filamentous fungi are more frequently detected in spoiled bread due to easier identification, spoilage yeasts such as *Hyp. Burtonii* remain a significant concern (Saranraj and Sivasakthivelan, 2015).

While molds are typically associated with spoilage in bakery products, certain fungal species have long played beneficial roles in traditional fermentations. For instance, *Aspergillus oryzae* is used industrially for enzyme production that can improve dough handling and baking performance, though it is more common in Asian food fermentations (Chang and Ehrlich, 2010; Machida et al., 2008). In bakery systems, the primary fermentative agents are yeasts and lactic acid bacteria (Erten et al., 2014). In addition to *Saccharomyces cerevisiae*, non-conventional yeasts such as *Candida milleri* (now *Kazachstania humilis*), *Kazachstania exigua*, *Torulaspora delbrueckii*, and *Wickerhamomyces anomalus* have been frequently isolated from sourdough and artisanal bakery environments (Ceresino et al., 2024). To manage spoilage without disrupting beneficial fermentative yeasts, selected LAB strains are increasingly used as natural preservatives, producing antifungal compounds that inhibit molds while supporting desirable sourdough microflora (Pérez-Alvarado et al., 2022). These yeasts contribute to dough acidification, enhanced aromatic complexity, and desirable textural properties. However, uncontrolled fungal growth remains a major concern.

Mycotoxins such as aflatoxins, ochratoxin A (OTA), and patulin, commonly associated with moldy grains and baked products, are linked to hepatotoxic, nephrotoxic, immunosuppressive, and carcinogenic effects. OTA, for example, is a potent nephrotoxin and possible human carcinogen (Group 2B, IARC) (Nazareth et al., 2024) The European Union has established maximum levels for OTA in cereals and cereal products at 3 μg/kg (European Commission, 2006). In the United States, although limits are less specific for bakery products, the FDA recommends limits for total aflatoxins in human food at 20 μg/kg (FDA, 2021).

Fungi in dairy products

Milk and dairy products are known for their lower susceptibility to spoilage compared to other food items, such as fruits or vegetables, because of thermal treatment processes, such as pasteurization and subsequent refrigerated storage. Despite these protective measures, a significant number of yeast and mold species exhibit a remarkable ability to survive and thrive in these environments (Garnier et al., 2017). This resilience can be attributed to the remarkable adaptation capabilities of fungi, which enable them to utilize a wide range of substrates found in dairy products, including lipids, organic acids, carbohydrates, and proteins. Consequently, their presence can cause changes such as visible fungal growth, off-flavors, and odors, as well as alterations to the color and texture of the products (Bento de Carvalho et al., 2024).

Thus far, up to 100 mold species have been shown to contribute to dairy product deterioration (Garnier et al., 2017). *Penicillium* species are among the most prevalent, followed by *Aspergillus*, *Mucor* and various yeast genera (Garnier et al., 2017). These fungi are the main contaminants in dairy products, leading to considerable annual food waste and economic setbacks on a global scale (Garnier et al., 2017). For instance, industry estimates suggest that even a 1.5–2% reduction in milk yield due to mycotoxin exposure in dairy cows can lead to over $15,000 in annual losses for a 200-cow farm producing 8,500 liters per lactation (Additive, 2024). Moreover, the risk of mycotoxins, such as aflatoxin B (hepatotoxic and carcinogenic), roquefortine C (neurotoxic), citrinin (nephrotoxic), and ochratoxin A (both nephrotoxic and potentially carcinogenic), carries potential health hazards. This shows the challenge of managing fungal contamination in dairy products (Hymery et al., 2014). Aflatoxin M1 (AFM1), a metabolite of AFB1, is particularly concerning in dairy due to its heat stability. Chronic dietary exposure to AFM1 has been linked to hepatocellular carcinoma, especially in individuals co-infected with hepatitis B virus (Liu et al., 2012). The EU sets the maximum level for AFM1 in milk at 0.05 μg/kg, while the US FDA permits up to 0.5 μg/kg (FDA, 2019; EU, 2006). Additionally, Roquefortine C and citrinin, produced by certain *Penicillium* species in cheese, are known for their neurotoxic and nephrotoxic effects, although regulatory thresholds for these compounds remain less well defined (Finoli et al., 2001).

Fungal contamination in dairy products can occur at any stage from dairy farms to after reaching the consumer's home, with sources ranging from unsanitary conditions to contaminated equipment and the addition of non-dairy ingredients (Garnier et al., 2017). The asexual spores (conidia) and vegetative cells of most mold species, along with yeasts, are sensitive to heat and typically do not survive pasteurization. However, they can cause food spoilage by producing heat resistant sexual ascospores and mycotoxins (Dagnas and Membré, 2013; Garnier et al., 2017; Pitt and Hocking, 2009). However, a small group of yeast species, *Debaryomyces, Saccharomyces*, and *Candida*, can survive heat processing and may cause spoilage of dairy products such as cheese and yogurt (Awasti and Anand, 2020). *Hamigara, Penicillium, Aspergillus,* and *Fusarium* are molds isolated from heat-treated dairy products, including cream cheese and pasteurized milk. In these molds, the heat-resistant sexual spores (ascospores) are responsible for their survival during pasteurization, or contamination may occur post-pasteurization (Garnier et al., 2017; Pitt and Hocking, 2009). Mold contamination in dairy factories is commonly linked to airborne transmission, as spores, mycelium fragments, and debris can readily spread through the air within these facilities. As demonstrated by Kure et al. (2004), air was identified as the primary carrier of significant cheese contaminants, such as *Penicillium commune* and *Penicillium palitans*, throughout the production process (Kure et al., 2004). To prevent airborne contamination, strategies such as high-efficiency particulate air (HEPA) filtration, positive air pressure systems, regular air quality monitoring, and strict sanitation protocols can be implemented to limit spore dispersion and accumulation in production areas (Garnier et al., 2017). Adding ingredients such as sweeteners, nuts, or fruits to dairy products, such as yogurt, can increase the risk of fungal spoilage by supplying additional sources of contamination and of nutrients that promote fungal growth and fermentation. Specifically, fruit additives, including blueberries and strawberries, are more susceptible to contamination because they cannot undergo extensive heat treatment

and could contain fungi capable of forming heat-resistant spores (Penney et al., 2004). *Debaryomyces hansenii*, is difficult to control in fruit-flavored yogurt products, due to its high osmotolerance, resistance to low pH, and ability to grow at refrigeration temperatures. Its presence can cause defects in flavor, texture, odor, and color (Pilote-Fortin et al., 2021).

Notably, certain fungi contribute positively to dairy products. For example, *Penicillium camemberti* and *Penicillium roqueforti* are essential in ripening cheeses such as Camembert and Roquefort, contributing to their unique aroma and flavor (Chávez et al., 2012). Nevertheless, the proliferation of unwanted species can lead to spoilage or mycotoxin contamination (Kure and Skaar, 2019). LAB-based biopreservation strategies offer a targeted way to suppress spoilage fungi while maintaining the activity of beneficial mold cultures used in ripened cheeses (Shi and Maktabdar, 2022).

Fungi in fruits and vegetables

Globally, between 20 and 30% of harvested fruits and vegetables are wasted each year, mainly due to decay caused by fungal contaminations occurring both pre-and post-harvest (Petrasch et al., 2019). More broadly, fungal plant pathogens are responsible for the destruction of up to 30% of total crop yield and contaminate approximately 25% of agricultural raw materials with spoilage fungi and mycotoxins (Nasrollahzadeh et al., 2022). These microorganisms predominantly follow necrotrophic or saprotrophic life cycles, catalyzing the decomposition of plant tissues either in the field, during harvesting, or post-harvest, ultimately resulting in the deterioration of the marketability of agricultural products. The production of mycotoxins by these fungi not only contaminates crops but also complicates food storage and preservation by requiring stringent measures to prevent further fungal growth and toxin accumulation (Bano et al., 2023).

Fungal infections in fruits involve four stages: spore adhesion, secure attachment, tissue invasion, and spread (Filippovich and Bachurina, 2022). In response, fruits activate antifungal defenses, which involve boosting phytohormone production, triggering an oxidative response, activating enzymes related to defense, and increasing the production of proteins that combat pathogens (Apaliya et al., 2017). Environmental parameters, both intrinsic (such as water availability, substrate composition, and pH) and external (such as humidity, temperature, and water activity), along with surrounding microorganisms, influence fungal infections and spore production (Bano et al., 2023). These factors collectively affect every stage of fungal growth, from spore germination to mycotoxin formation and mycelial development. Even with elevated water activity in vegetables and fruits, the relatively low pH, particularly in fruits, creates an environment favoring fungi over bacteria, leading to common mold spoilage (Bueno et al., 2007; Tournas, 2005).

The major fungi responsible for fruit and vegetable spoilage belong to the genera *Alternaria, Penicillium, Aspergillus,* and *Fusarium*. The mycotoxins they produce are Alternaria toxins (ATs), patulin (PAT), trichothecenes (TCs), and ochratoxin A (OTA). *Alternaria alternata* from the *Alternaria* genus causes mycotoxin contamination in a variety of crops, such as apples, strawberries, pears, melons, citrus, tomatoes, and potatoes (Logrieco et al., 2009). *Aspergillus* species, especially *A. flavus*, *Aspergillus niger*, and *Aspergillus ochraceus*, can infect plant tissues and produce certain types of mycotoxins, including fumonisins, aflatoxins, patulin, and ochratoxin A, which are found in seeds and fruits (Sanzani et al., 2016). *A. niger* is the predominant species causing decay in harvested fruits, such as citrus, apples, pears, peaches, grapes, figs, and strawberries. While spoilage in these fruits is often considered minor in terms of economic or health impact, the spoilage of crops such as tomatoes, onions, and garlic can lead to more significant postharvest losses under certain conditions (Plascencia-Jatomea et al., 2014). Most research focused on mycotoxins in fruits primarily centered around the toxin patulin, mainly synthesized by *Penicillium expansum* (derived from apples), and ochratoxin A, predominantly produced by *Aspergillus carbonarius* (derived from grapes and wines) (Zhang et al., 2017). Patulin contamination is a significant concern, considering that many apple-based processed foods are destined for infant nutrition (Sarubbi et al., 2016). Patulin exhibits genotoxic and immunotoxic effects. The EU and WHO set maximum permitted levels of patulin at 50 μg/kg for fruit juices and 10 μg/kg for baby foods (European Commission, 2003). OTA is frequently detected in grape-derived products and is considered a possible human carcinogen. *Aspergillus carbonarius*, prevalent in vineyards, is a major contributor to OTA accumulation during postharvest handling. The EU limit for OTA in grape juice and wine is 2 μg/kg (European Commission, 2006). Pathogens that cause decay in the storage phase often come from fields or orchards. The spores of mycotoxigenic isolates are found in the fruits of trees, yet they usually do not initiate growth or mycotoxin production until post-harvest (Sarrocco and Vannacci, 2018). Ochratoxin A is a globally recognized contaminant in grapes, wine, and other grape-derived products, such as juice or must. Black *Aspergilli*, especially *A. niger* and *A. carbonarius*, are common in vineyards-acting as primary contributors to the production of this toxin in grapes. During the postharvest phase, fungal species such as *A. carbonarius* become dominant, with *A. carbonarius* recognized as the most potent producer of ochratoxin A (OTA) in grapes (Sanzani et al., 2016).

Despite continuous efforts to eliminate mycotoxins, their presence in agricultural produce remains unavoidable. To reduce the risk of mycotoxin production, dissemination, and mold growth, it is necessary to develop and implement effective strategies that include three major components: Hazard Analysis Critical Control Points, Good Agricultural Practices, and emerging methods such as non-thermal preservation technologies and biological control approaches. These elements are key to minimizing the presence and impact of mycotoxins in food and feed (Paster and Barkai-Golan, 2008).

While fungal contamination in fruits and vegetables is typically associated with spoilage, certain fungi also serve protective roles. Species such as *Trichoderma harzianum* and *Aureobasidium pullulans* are used in postharvest biocontrol of fruits such as apples, grapes, and strawberries, where they suppress pathogens such as *Penicillium expansum* and *Botrytis cinerea* through competition, antibiosis, and mycoparasitism (Ippolito et al., 2000; Yao et al., 2023). In addition to fungal biocontrol agents, LAB-derived coatings and metabolites have shown promise in reducing surface spoilage fungi, thereby extending shelf life without affecting the fruit's microbial balance (Ranjith et al., 2022).

Fungi in meat products

Although the muscles of healthy animals are generally considered sterile, meat and meat products are susceptible to contamination

during all stages of slaughter, preparation, and processing. Thus, the microbial ecosystem of meat and meat products is rich and diverse (Chaillou et al., 2015). The availability of nutrients, high water activity (nearly 0.99), and pH of 5.5 present favorable conditions for the propagation of a variety of microorganisms. Immediately after slaughter, most bacteria found on the carcasses are Gram-positive, of which 99% are mesophilic. With increasing storage time and low storage temperature, Gram-negative psychrotrophic bacteria gradually become dominant under aerobic conditions. When anaerobiosis sets in gradually, *Lactobacillus* and other facultative aerobic microorganisms, such as *Enterobacter* and *Brochothrix*, as well as fungi and mold, dominate (Hazards et al., 2023).

The fungi and molds proliferate in processed meat products such as fermented, dry-cured, or frozen meat (Mastanjević et al., 2023). Because of fermentation, ripening stages, and handling conditions, the physicochemical properties of these products become more suitable for contamination with a variety of beneficial and undesirable fungi and molds. Beneficial fungi improve desirable food properties by enhancing flavor quality through the secretion of specific enzymes such as lipases and proteases. Beneficial species such as *Penicillium nalgiovense* and *D. hansenii* contribute to flavor and surface protection, while undesirable fungi such as *Penicillium commune*, *A. flavus*, and *Mucor* spp. are associated with spoilage, off-odors, discoloration, or mycotoxin production (Hernandez-Mendoza et al., 2009). Others are toxigenic fungi and molds, which lead to undesirable odors and flavors, spoilage, and mycotoxin contamination. The growth of spoilage molds and yeasts in meat products is highly influenced by temperature and humidity. Most fungi, including *Penicillium* and *Cladosporium* species, thrive at temperatures between 15–30°C and relative humidity above 85%, particularly during the ripening and storage of dry-cured meats (Alves Rodrigues et al., 2025). Uncontrolled humidity and temperature in storage or processing facilities can accelerate spoilage and increase the risk of visible mold formation or mycotoxin production.

Fungal ecosystems of meat products are rich and diverse. A myriad of yeast species are obtained from meat products undergoing fermentation, such as *Rhodotorula mucilaginosa*, *D. hansenii*, *Cryptococcus* strains and *Candida* genus. It was also shown that this yeast population was dominated by *Yarrowia lipolytica*, *Candida zeylanoides* (a synonym of *Debaryomyces hansenii* according to some classifications) and basidiomycetous yeasts. While some of these yeasts, particularly *D. hansenii* and *C. zeylanoides*, play beneficial roles in flavor development, lipid breakdown, and surface stabilization during fermentation and ripening, others such as *Y. lipolytica* may contribute to spoilage under suboptimal storage conditions. In dry-cured Parma ham, Simoncini et al. (2007) identified a yeast population dominated by *D. hansenii*, *C. zeylanoides*, *Debaryomyces maramus*, *Hyphopichia burtonii,* many of which are considered technologically important for flavor and texture development in artisanal meat products (Simoncini et al., 2007). While meat is less commonly associated with dietary mycotoxin exposure, fungal metabolites such as OTA and citrinin can be introduced through contaminated spices or additives (Pleadin et al., 2021). OTA is a concern due to its stability and has been found in dry-cured meats (Toman et al., 2024). The EU limits OTA in meat products are not universally established, but stricter controls exist for spices (e.g., 15 μg/kg in nutmeg and paprika), which are often added to processed meats (Leprêtre and Merten-Lentz, 2018). Although financial estimates specific to fungal spoilage in meats are limited, case studies have shown OTA contamination rates of up to 100% in dry-cured hams from both industrial and household production sources (Pietri et al., 2011). Such contamination can lead to product rejection, recall, and reputational damage, underscoring the need for robust fungal control measures (Chen et al., 2022).

On the beneficial side, surface-ripened fermented meats often rely on intentional fungal colonization (such as *Penicillium nalgiovense*) to prevent oxidation and protect against spoilage organisms (Bernáldez et al., 2013). LAB strains used in dry-cured meats also contribute to microbial stability by producing organic acids and bacteriocins, which inhibit spoilage fungi while preserving the function of surface molds such as *Penicillium nalgiovense* (Laranjo et al., 2019).

Food mycotoxins and their health effects

Mycotoxins are resistant to many microbiological food stabilization techniques, such as heating (Oliveira et al., 2015). As a result, humans and animals that ingest tainted food and feed are exposed to the toxic effects of these toxins. However, some mycotoxins possess significant antibiotic properties, which can exert selective pressure on microbial populations, promoting the emergence of resistant bacterial strains. This is particularly concerning in the gut microbiota of humans and animals consuming contaminated food, as it may reduce the efficacy of clinically important antibiotics such as penicillin and disrupt microbial balance (Modi et al., 2014). Mycotoxins can contaminate on many types of foods, such as fruits/dried fruits, nuts, spices, cereals, grains, and cheese at any point during the storing, harvesting, or production phase (Patel et al., 2021). Their occurrence is influenced by factors such as high humidity, elevated temperatures, insect damage, and poor storage conditions, which create favorable environments for fungal growth and toxin production.

A few 100 mycotoxins have been identified, with approximately 30 of them found in mold-contaminated food and feed (Zhang et al., 2016). The main foodborne mycotoxins of public health concern are fumonisins, aflatoxins, trichothecenes, zearalenone, and ochratoxin A, although there are many others (Nguegwouo et al., 2018; Wu et al., 2014). Mycotoxins have been linked to mild and long-term human illnesses and can cause cancer in various organs, such as the liver, lungs, and kidneys, illustrating that harmful foodborne mycotoxins can affect human health (Figure 1).

Numerous studies have shown that foodborne mycotoxins can induce both acute and chronic toxicity, with carcinogenic, nephrotoxic, hepatotoxic, immunotoxic, teratogenic, and neurotoxic effects in humans and animals (Milićević et al., 2010). For instance, aflatoxin B1, produced by *A. flavus* and *A. parasiticus*, is one of the most potent naturally occurring liver carcinogens and has been classified by the International Agency for Research on Cancer (IARC) as a Group 1 carcinogen (Mohd Redzwan et al., 2016). Its carcinogenic effect is primarily due to the formation of DNA adducts, particularly AFB1–8,9-epoxide, which binds to guanine residues in DNA, leading to mutations in the TP53 tumor suppressor gene, a hallmark event in hepatocellular carcinoma (Bedard and Massey, 2006). Trichothecenes (e.g., T-2 toxin and deoxynivalenol) interfere with ribosomal function, inhibiting protein synthesis and triggering apoptosis, immunosuppression, and gastrointestinal distress (Hooft and Bureau, 2021).

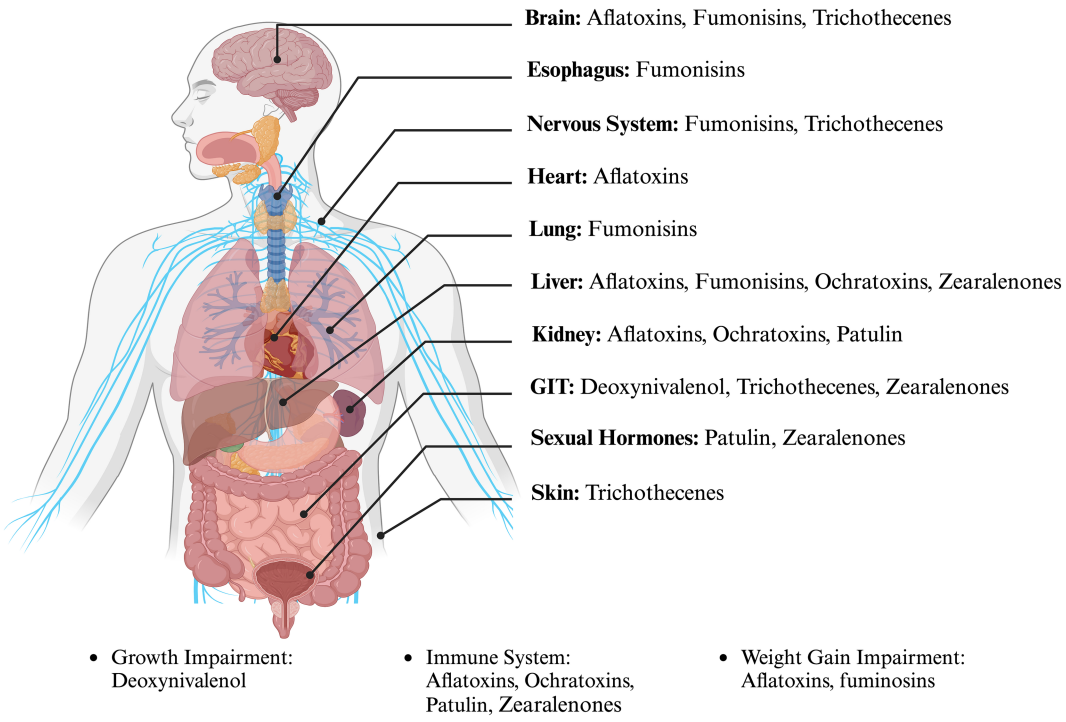

FIGURE 1
The most common foodborne mycotoxins and their impact on human organs. (Created with BioRender.com).

Mycotoxins are prevalent in various foods, including some fermented and ripened products where molds may develop during processing or storage (Chelule et al., 2010). According to Marín et al. (2018), oilseeds, cereals, dried fruits, spices, flour, milk products, coffee, and other by-products are primary commodities that facilitate fungal growth and mycotoxin production. While there is overlap with the commodities listed in Table 1 (e.g., cereals, dried fruits, and spices), this list also includes additional categories such as oilseeds, flour, and milk products, suggesting that fungal contamination and mycotoxin risks extend across a broader range of food products (Marín et al., 2018). The most prevalent fungus in cheese is *Penicillium*, and *Penicillium expansum* produces patulin, a carcinogen that is more potent than the heterocyclic aromatic amines citrinin, polycyclic aromatic hydrocarbons, and nitrosamines. Fumonisins are produced mainly by *Fusarium* species, which can grow well on maize and foods made with maize is thought to cause swelling and throat cancer (Smith, 2018). Fumonisin disrupts sphingolipid metabolism by inhibiting ceramide synthase, a critical enzyme for maintaining cell membrane integrity and signaling. This mechanism has been associated with esophageal cancer and neural tube defects in high-exposure regions such as parts of China and South Africa (Voss et al., 2002). Zearalenone, another mycotoxin produced by *Fusarium* spp., mimics estrogen and binds to estrogen receptors, leading to reproductive disorders and potential endocrine disruption, in individuals and livestock (Sajjad et al., 2025). In grains, *Aspergillus* and *Penicillium* species are primarily responsible for producing ochratoxins. *A. parasiticus* and *A. flavus* are the two major producers of aflatoxin, and both prefer milk products as substrates (Makau et al., 2016). Ochratoxin A exhibits nephrotoxicity and has been linked to Balkan endemic nephropathy and renal tumors. The toxicity of OTA is associated with oxidative stress, inhibition of protein synthesis, and DNA damage, though its carcinogenic classification remains under IARC Group 2B (possible human carcinogen) (Bui-Klimke and Wu, 2015). Table 1 summarizes the most commonly encountered foodborne mycotoxins, the fungi that produce them, and the food products that harbor them.

Preventing mold growth: LAB as a valuable source of antimicrobial compounds

LAB have antimicrobial capabilities that hinder the proliferation of fungi and various Gram-positive and Gram-negative bacteria, justifying their application in food fermentation, preservation, and storage. LAB can produce a range of antimicrobial substances including organic acids, reuterin, hydrogen peroxide, hydroxylated fatty acids, exopolysaccharides, and bacteriocins. These compounds exhibit distinct modes of action that contribute to microbial inhibition and shelf life extension in food systems (Figure 2).

Among the low molecular weight metabolites, organic acids such as lactic, acetic, and propionic acids acidify the food matrix and reduce pH, creating unfavorable conditions for fungal growth. Diacetyl interferes with microbial metabolism by reacting with amino groups, while hydrogen peroxide induces oxidative stress via reactive oxygen species that damage fungal membranes and DNA (Liu A. et al., 2022). Reuterin, produced by *Limosilactobacillus reuteri*, is a broad-spectrum antimicrobial that functions by alkylating thiol groups in proteins and enzymes of spoilage organisms. While its synergy with organic acids such as lactic acid has been demonstrated against bacteria, whether this interaction enhances antifungal activity remains to be confirmed (Soltani et al., 2022).

TABLE 1 Common mycotoxins in food products and fungal species associated with their production.

Food source	Mycotoxins	Fungal species	References
Cereals	Aflatoxins	*A. flavus, A. parasiticus*	Abbès et al. (2016), Chen et al. (2018)
	Ochratoxins	*A. ochraceus, A. carbonarius, A. niger*	JØrgensen (2005), Szőke et al. (2022)
	Zearalenone	*F. graminearum, F. culmorum, F. cerealis*	Ghazvini et al. (2016), Shephard (2008)
	Trichothecenes	*F. graminearum, F. culmorum*	Beasley (1989), Woloshuk and Shim (2013)
Dairy and eggs	Aflatoxins	*A. flavus, A. parasiticus*	Embaby et al. (2022), Taniwaki et al. (2018)
Fruits and juices	Ochratoxins	*A. ochraceus, A. carbonarius, A. niger*	Serra Bonvehí (2004), Zimmerli and Dick (1996)
	Patulin	*Penic. expansum*	Nan et al. (2022), Wright (2015)
Meat	Aflatoxins	*A. flavus, A. parasiticus*	Abbès et al. (2016), Chen et al. (2018)
Nuts and dried fruits	Aflatoxins	*A. flavus, A. parasiticus*	Embaby et al. (2022), Taniwaki et al. (2018)
Vegetables	Aflatoxins	*A. flavus, A. parasiticus*	Chen et al. (2018), Embaby et al. (2022)
Maize and processed products	Fumonisins	*Fusarium verticillioides, F. proliferatum*	Blanchard and Manderville (2016), JØrgensen (2005)
Wine and coffee	Ochratoxins	*A. ochraceus, A. carbonarius, A. niger*	Blanchard and Manderville (2016), JØrgensen (2005)
Spices and beans	Ochratoxins	*A. ochraceus, A. carbonarius, A. niger*	Serra Bonvehí (2004), Szőke et al. (2022)

Penic, Penicillium.

Some LAB strains, notably *Lactiplantibacillus plantarum,* can hydroxylate unsaturated fatty acids to produce hydroxy derivatives such as 3-hydroxytetradecanoic acid and 3-hydroxydodecanoic. These compounds exhibit antifungal properties by integrating into fungal membranes and disrupting their integrity (Sjögren et al., 2003). These molecules are particularly effective against food spoilage yeasts and molds in low-pH environments typical of fermented foods (Nasrollahzadeh et al., 2022).

Extracellular polymeric substances (EPS), produced by certain LAB strains, are multifunctional secondary metabolites. While primarily known for improving food texture and mouthfeel (biothickening), some EPS also exhibit antiadhesive or biofilm-inhibitory properties, reducing the ability of fungi to colonize surfaces in food systems (Kavitake et al., 2023). In addition, EPS may indirectly contribute to antifungal activity by promoting LAB competitiveness and persistence in food environments. EPS from LAB are also known to stimulate immune responses and modulate gut microbiota, offering dual functionality in health and preservation (Jurášková et al., 2022).

Bacteriocins, another important group of LAB metabolites, are ribosomally synthesized proteinaceous molecules produced by LAB that primarily target bacteria. Although they are predominantly known for their antibacterial activity, certain strain-specific bacteriocin-like inhibitory substances (BLIS), such as those produced by *Pediococcus pentosaceus*, have been reported to exhibit antifungal or antimycotoxigenic effects in specific contexts, particularly in silage and feed matrices (Dalié et al., 2010; Souza De Azevedo et al., 2020). However, the mechanisms responsible for these effects remain insufficiently studied, and such activity is not consistently observed across LAB strains or food systems. Nisin, produced by *Lactococcus lactis*, is the most extensively studied and the only bacteriocin currently approved for use in food. It is widely applied in dairy and meat products to control spoilage and pathogenic bacteria. Nisin acts by binding to lipid II, a precursor in bacterial cell wall synthesis, leading to pore formation and cell lysis (Field et al., 2023). While primarily active against Gram-positive bacteria, some studies suggest nisin may also inhibit mold spore germination or indirectly reduce fungal colonization by suppressing associated bacteria that facilitate fungal growth (Gut et al., 2008). Bacteriocins are considered promising biopreservatives due to their neutral sensory profile and stability across a broad range of pH, temperature, and salt conditions. They can be delivered as bioactive powders or incorporated into antimicrobial packaging, offering extended protection without altering food quality. Limitations such as narrow activity spectrum or inactivation by food components may be addressed through encapsulation techniques or combined preservation strategies (Bastos et al., 2015).

Antifungal compounds from LAB

The family Lactobacillaceae is one of the dominant groups in the food microbiome and is strongly associated with antifungal activity. Extending shelf life while maintaining stability and safety in foods is the primary objective, typically achieved by inhibiting pathogenic microorganisms and spoilage. Various antimicrobial agents may be used alone or synergistically to inhibit the proliferation of spoilage microorganisms and foodborne pathogens. Importantly, these agents preserve the nutritional and sensory characteristics of foods, maintaining their physicochemical structure. In addition to their antifungal properties, LAB are beneficial in food products as they can: (1) limit the proliferation of hazardous enteric pathogens, (2) provide beneficial enzymes, (3) eliminate toxic food components in the gut, (4) enhance the immune system, and (5) stimulate peristaltic movement of food through the gastrointestinal tract. Table 2 summarizes the LAB strains obtained from various sources, along with their antifungal activity spectra. Membrane instability, enzyme inhibition, the formation of oxygen-reactive species, and proton gradient interference (Schaefer et al., 2010) are some of the mechanisms behind the inhibition of single compounds that have been explored in depth (Figure 2). However, many investigations have focused solely on the effects of individual compounds, ignoring the synergistic and additive effects of compounds used in combination. Future studies should explore optimized combinations of LAB-derived metabolites, their mechanisms of interaction, and their efficacy in complex food matrices under real storage conditions.

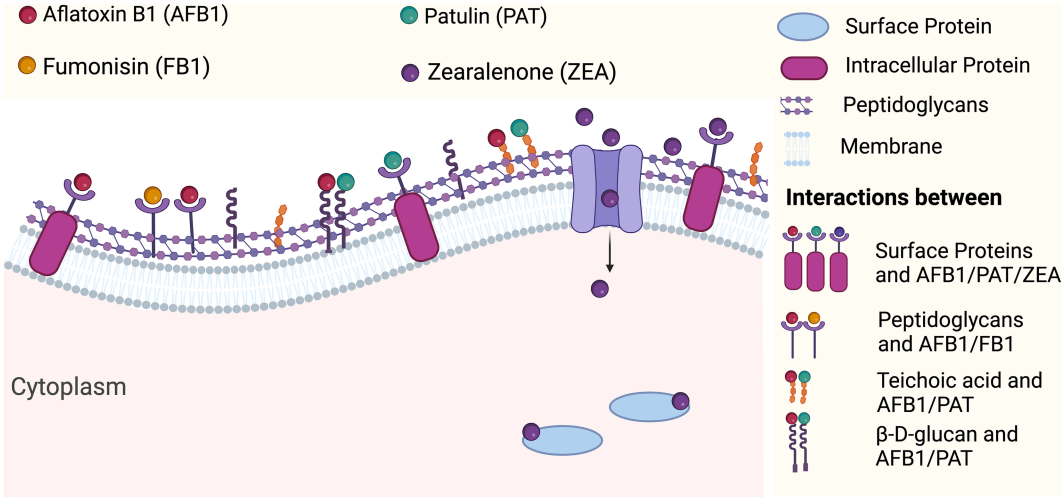

FIGURE 2
Antimicrobial compounds from LAB strains and their inhibitory effects on spoilage microbes. (Created with Biorender.com).

TABLE 2 Antifungal activity spectrum of lactic acid bacteria sourced from a diversity of food and feed.

Strain	Source	Targets	Action	Active compound	References
Lcb. casei; L. delbrueckii spp.	Dairy	*Penicillium* spp. *Diutina rugosa*	Proteolytic activity; Unknown	CFS	Erginkaya et al. (2004), Girardin (1997)
L. helveticus		*Penicillium* sp.	Proton gradient interference	LA, AA	Bian et al. (2016)
Lcb. rhamnosus MDC 9661; Sch. harbinensis K. V9.3.1 Np		*Various fungi, including Penic. thomii*	Linked with cell wall: Yeast membrane depolarization	LBC; LA, CFS	Bazukyan et al. (2018), Mieszkin et al. (2017)
Liml. fermentum (Cassava, Andean products)	Fermented Products (Non-Dairy)	*Aspergillus* spp., *Penic. expansum*	Unknown; Proton gradient interference	CFS; PLA, 3,5-Di-O-caffeoylquinic acids	Adedokun et al. (2016), Yépez et al. (2017)
Liml. fermentum C14 (Curd)		*Penic. digitatum, Mucor* sp.	Proton gradient interference	LA, PLA, CFS	Barman et al. (2017)
Fruct. sanfranciscensis CB1; Lcb. paracasei subsp. *tolerans*	Sourdough	*Various fungi including Fusarium, Penicillium*	Unknown	Mixture of organic acids	Corsetti et al. (1998), Hassan and Bullerman (2008)
Furfl. rossiae; Liml. reuteri CRL 1100, Levil. brevis CRL 772/796		*A. niger, Penic. roqueforti; Aspergillus, Fusarium*	Proton gradient interference	LA, AA, PLA	Gerez et al. (2009), Valerio et al. (2009)
Secund. paracollinoides	Vegetable	*F. graminearum, Botrytis cinerea*	Unknown	LBC	Sathe et al. (2007)
Liquor. mali VLT112, Lcp. pentosus VLT310 (Salami)	Meat	*A. candidus, Penic. nalgiovense*	Proton gradient interference, enzyme inhibition	PLA, HO-PLA	Corsetti and Settanni (2007)
Luig. coryniformis Si3	Grass silage	Various fungi including *F. sporotrichoides*	Unknown	Partially purified compounds	Magnusson and Schnürer (2001)
Liml. fermentum	Cocoa bean	*Penic. citrinum, G. moniliformis*	Proton gradient interference	AA, LA	Romanens et al. (2019)

AA, Acetic acid; LA, Lactic acid; BA, Butyric acid; PA, propionic acid; FA, Formic acid; PLA, Phenyllactic acid; SA, Salicylic acid; LBC, Live bacterial cell; CFS, Cell free supernatant; PCO, Proteinous compound; UN, Unknow; Penic, Penicillium.

Mycotoxin detoxification by LAB species

Mycotoxin detoxification by LAB species involves detoxification of various mycotoxins. Biological detoxification uses microorganisms and metabolites to biotransform mycotoxins into less harmful or harmless chemicals (Liu L. et al., 2022). Biological detoxification is a promising option because hundreds of microorganisms and metabolites are available. The microorganisms used for detoxification must be safe, and capable of producing stable, nontoxic metabolites that degrade mycotoxins into harmless byproducts through irreversible chemical reactions. Additionally, they must avoid generating undesirable odors or tastes, possess minimal cultivation and production requirements, remain active throughout storage, and preserve the nutritional value of the food. Several microbes have been

proposed as food and feed detoxifiers, but only a few have been tested for practical use. Yeast, bacteria, and fungi are the microorganisms most commonly employed to detoxify feed and food (Shekhar et al., 2025). Karlovsky (1999) explored the prospect of using microbes such as *Rhizopus* sp., *A. niger*, *Yarrowia lipolytica* (formerly *Candida lipolytica*), *Mucor ambiguus, Neurospora* spp., *Trichoderma viride, Desarmillaria tabescens* (formerly *Armillariella tabescens*), and LAB for detoxification (Karlovsky, 1999). Owing to their established safety, LAB are the preferred microorganisms for degrading mycotoxins. LAB are chosen over other microbes because they are recognized as safe, occur naturally in the human digestive tract, and remain easy to cultivate and maintain. Lactic acid bacteria follow two pathways for food mycotoxin detoxification: (1) using the viable cells of microorganisms and (2) enzymes produced by specific LAB strains. Lactic acid bacteria proteolytic enzymes play important roles in detoxifying mycotoxins in food (Nichea et al., 2015; Wu et al., 2009). The use of LAB to reduce mycotoxins in food has been investigated extensively (Table 3). Integrating LAB cells and their metabolites to reduce mycotoxin levels in food offers several benefits. The adsorption of mycotoxins by the LAB cell wall has been proposed as a potential strategy for mycotoxin removal from food. This action involves polysaccharides, proteins, and cell walls of LAB strains that contain peptidoglycans (Chapot-Chartier and Kulakauskas, 2014). The binding activity of specific LAB strains has been shown to decrease patulin levels in culture media (Wang et al., 2015). This study suggests that LAB strains with thicker cell walls and greater surface areas can improve their binding to and neutralization of patulin, leading to mycotoxins exclusion. Polysaccharides and proteins have been reported to be critical functional components for the adsorption of patulin. Another study concluded that mycotoxin binding by LAB cells was dependent on the initial mycotoxin concentration, LAB cell count, food complexity, pH, and incubation temperature (Luz et al., 2018). Research conducted by Dalié et al. (2010) showed that living bacterial cells in yogurt are not essential for the detoxification of aflatoxin B1. This is because aflatoxin B1 binds to specific components on the cell wall from dead bacteria, such as polysaccharides or proteins, facilitating its neutralization in yogurt (Dalié et al., 2010).

Metabolites produced by LAB strains, such as acids, low-molecular-weight bioactive peptides, phenolic compounds, and fatty acids, interact to reduce the levels of mycotoxins in foods (Niderkorn et al., 2009). These metabolites contribute to detoxification through diverse mechanisms, such as enzymatic degradation and chemical interactions with mycotoxins. The breakdown of mycotoxins and their elimination by metabolites and LAB cells are not yet fully understood (Muhialdin et al., 2020). However, hypotheses regarding various mechanisms have been proposed, including proteolytic enzyme activity and specific interactions between metabolites and mycotoxins. These interactions often lead to binding, sequestration, and in some cases, degradation of mycotoxins. For example, proteolytic enzymes may hydrolyze mycotoxin structures, while specific cell wall components, such as peptidoglycans, surface proteins, and teichoic acids, facilitate binding and potentially enhance enzymatic degradation (Liu L. et al., 2022) (Figure 3). Studies have demonstrated that LAB enzymes, cells, and metabolites can work simultaneously to degrade or neutralize mycotoxins, reducing their toxicity and prevalence in food systems (Muhialdin et al., 2020).

Lahtinen's research group discovered that the binding of AFB1 by *Lacticaseibacillus rhamnosus* strain GG, is linked to cell wall peptidoglycans, emphasizing the importance of bacterial cell wall components in mycotoxin adsorption (Lahtinen et al., 2004). AFB1 binding is not associated with exopolysaccharides, minerals (calcium or magnesium), proteins, or lipids (Lahtinen et al., 2004). Similarly, teichoic acids from *Lacticaseibacillus casei* Shirota and *Liml. reuteri* NRRL14171 have been found to bind AFB1 (Hernandez-Mendoza et al., 2009). The binding ability of *Lcb. casei* Shirota cells to AFB1 is attributed to proteins on the bacterial cell wall. AFB1 may also attach

TABLE 3 Mycotoxin detoxification by LAB species or strains.

Target mycotoxin	Microorganism (species or strain)	Degradation (%)	Culture form	References
Aflatoxin (AFB1, AFB2, AFG1, AFG2)	*L. delbrueckii*	>99%	LBC	Saladino et al. (2016)
AFB1	*L. amylovorus; Levil. brevis LOCK 0944 (Note: This appears twice in the list) Lent. buchneri; L. bulgaricus; Lcb. casei; Lcb. casei LOCK 0920; Lcb. paracasei LOCK 0920; Lpb. plantarum; Lpb. plantarum C88; Lpb. plantarum LOCK 0945 (Note: This appears twice in the list); Lpb. plantarum MYS44; Lpb. plantarum Lcb. rhamnosus; Lcb. rhamnosus GG; Lcb. rhamnosus LC-705; Lpb. plantarum Lactococcus lactis Pedioc. acidilactici; S. thermophilus*	11–81%	LBC/CFS	Poornachandra Rao et al. (2019), Sezer et al. (2013)
Ochratoxin (OTA)	*B. animalis* subsp. *lactis VM12; L. acidophilus VM 20; Levil. brevis LOCK 0944; Lent. buchneri; Lcb. casei LOCK 0920; Liml. fermentum; Lpb. plantarum; Lpb. plantarum LOCK 0945; Liml reuteri; Lcb. rhamnosus CECT 278 T; Pedioc. parvulus*	50–97%	LBC	Fuchs et al. (2008), Luz et al. (2018), Niderkorn et al. (2009)
Patulin	*Lent. buchneri;, Liml reuteri; Lpb. plantarum; Liml. fermentum; Enterococcus faecium; Lcb rhamnosus; L. acidophilus; Lpb. plantarum*	65–90%	LBC/HKBC	Hatab et al. (2012), Zoghi et al. (2017)
Zearalenone (ZEA)	*Pedioc. pentosaceus KTU05-9; Pedioc. acidilactici; Lcb. paracasei, L. lactis*	37–55%	LBC	Juodeikiene et al. (2018), Rogowska et al. (2019)

LBC, Live bacterial cell; CFS, Cell-free supernatant; HKBC, heat-killed bacterial cell; Pedioc, Pediococcus.

to the bacterial cell wall D-glucans through van der Waals interactions and the formation of hydrogen bonds (Yiannikouris et al., 2006).

While binding interactions are crucial for sequestering AFB1, they may also facilitate degradation by creating proximity to enzymatic or chemical processes that break down the toxin. Environmental factors, such as pH and temperature, influence the cell wall structure and physicochemical properties, including electrostatic interactions, thereby affecting the availability of mycotoxin binding sites (Muhialdin et al., 2020). Acidic conditions, for instance, enhances hydrophobic interactions by breaking down surface proteins and exposing additional AFB1 binding sites. These interactions not only favor adsorption but may also enhance degradation pathways, underscoring the importance of understanding how structural and environmental factors contribute to improving LAB efficiency in reducing mycotoxin toxicity (Bueno et al., 2007).

Patulin

Patulin is a water-soluble mycotoxin produced by numerous *Aspergillus* and *Penicillium* species. However, the apple rot fungus *Penicillium expansum* is the most prevalent species involved in patulin synthesis (Plascencia-Jatomea et al., 2014). High levels are found in foods, such as grapes and grains. Patulin residues are problematic because they can enter tissues, stop protein synthesis, and lower glycogen levels in the intestines, kidneys, and liver. The critical binding structures of patulin are cell surface adhesion proteins. Recent studies have identified various parameters associated with patulin biosorption by inactivated LAB strains. According to Wang et al. (2015), pretreatment with esterification and NaOH improved patulin binding. In contrast, pretreatment with iodate, trypsin, periodate, and lipase reduced binding (Wang et al., 2015). The significance of vicinal and carboxyl OH groups was negated, with esters, alkaline amino acids,

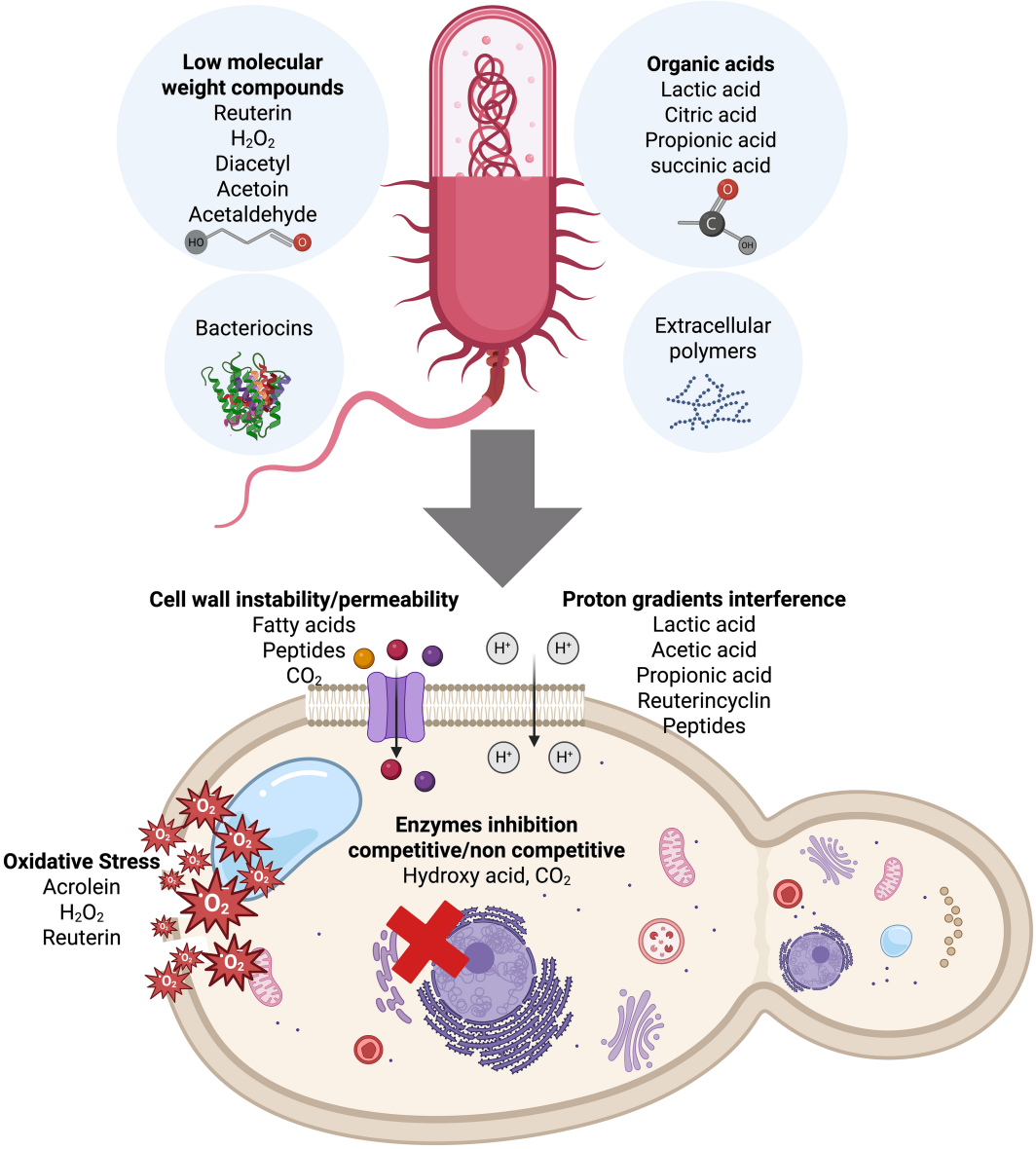

FIGURE 3
Mycotoxin interaction with bacterial cell walls. (Created with BioRender.com).

and thiol suggested as probable molecules implicated in adsorption. LAB strains adsorb patulin through hydrophobic and electrostatic interactions. Wang et al. hypothesized that greater patulin adsorption could be attributed to a larger cell area and volume, which varies among species and cell types based on differences in cell wall composition and structural characteristics. LAB carbohydrate components (NH, C-O, and OH groups) and cell surface proteins have also been identified as key factors influencing patulin adsorption (Wang et al., 2015). The information provided did not reveal the specific processes or interactions linking the bacterial cell wall attributes and mycotoxins (Figure 3). However, certain compounds, such as fructooligosaccharides and ascorbic acid, have been shown to enhance the patulin-binding capacity of *Lpb. plantarum* ATCC 8014 and *Lactobacillus acidophilus* ATCC 4356 by possibly altering the cell wall structure or increasing the availability of functional binding sites. This effect appears to be strain-dependent and may not be common across all strains within these species (Zoghi et al., 2017).

Fumonisins (FB)

Fumonisin is a mycotoxin primarily produced by *Fusarium proliferatum* and *Fusarium verticillioides* (formerly *F. moniliforme*). Among the fumonisins, B1, B2, and B3 are the most prevalent and toxic, with B1 being the most frequently detected and associated with significant health risks, such as carcinogenicity and disruption of sphingolipid metabolism (Anumudu et al., 2024). Fusarium-infected corn may sicken animals and induce equine leukoencephalomalacia (ELEM), a long-standing disease in North America. Fumonisins are found in corn, tortillas, sorghum, rice, and medicinal plants. Niderkorn found that fumonisin (FB1 and FB2) probably binds to peptidoglycan or molecules very similar to it without the help of surface lipids, proteins, or polysaccharides (Niderkorn et al., 2009) (Figure 3). The peptidoglycans of *Lpb. plantarum* B7 and *Lactiplantibacillus pentosus* X8 had maximum capacity to bind fumonisins compared to other LAB strains tested in the study, demonstrating their superior binding efficiency relative to the other strains evaluated (Zhao et al., 2016). The mechanism of fumonisin-LAB cell wall peptidoglycan interaction is unknown. The interaction of fumonisin tricarballylic acid chains occurs with peptidoglycans during binding (Niderkorn et al., 2009).

Zearalenone (ZEA)

Several *Fusarium* species produce zearalenone, a nonsteroidal estrogenic mycotoxin. *Fusarium graminearum* is the primary producer of ZEA. Some *Fusarium* species that produce zearalenone include *Fusarium sporotrichioides, Fusarium semitectum, Fusarium equiseti, Fusarium oxysporum, Fusarium culmorum,* and *F. verticillioides.* Contamination of cereal grains with zearalenone has been reported in warm climates (Milani, 2013). Generally, zearalenone concentrations are low in contaminated field grains, but tend to increase during storage, with moisture levels of 30–40% (Agriopoulou et al., 2020b). Zearalenone significantly affects female reproduction (hyperestrogenism) and male reproduction (Yang et al., 2007). Recent research indicates zearalenone poses serious human health risks (Belhassen et al., 2014; Mally et al., 2016). ZEA removal by LAB has been shown to be strain-dependent and influenced by factors such as the protein and lipid composition of the bacterial cell wall, bacterial concentration, and the presence of co-occurring mycotoxins, which may compete for binding sites. Heat and acid treatments have also been shown to significantly enhance ZEA and α-ZOL removal, while polysaccharides and pH appear to have limited effect (Adunphatcharaphon et al., 2021). From the initial adsorption stage of 720 min, this rate fell from 5.49 g/mL min^{-1} to 0.15 g/mL min^{-1} during the secondary adsorption, reaching equilibrium. LAB may remove ZEA through cell surface proteins, peptidoglycans, or absorption into bacterial cell interactions (Figure 3). Likewise, cell wall components of *Lpb. plantarum* strain 102 bind the T-2 toxin (Król et al., 2018). Trichothecene mycotoxin elimination by LAB strains is only known to occur through cell wall binding.

Antifungal activity of LAB in bakery products

The use of LAB as preservatives is extensive, as reviewed by (Crowley et al., 2013; Shi and Maktabdar, 2022; Zapaśnik et al., 2022). It has been demonstrated that incorporating LAB into sourdough fermentation is an effective strategy to prevent mold spoilage in bread, as LAB can produce antifungal metabolites during the fermentation process. This effect is partially due to the generation of organic acids such as acetic or lactic acid (Le Lay et al., 2016). Additionally, it has been shown that acidifying dough significantly influences the qualitative features of bread, such as volume and texture (Jekle and Becker, 2012). Among the various LAB strains used in sourdough, *Levilactobacillus brevis* and *Lpb. plantarum* have been shown to have favorable effects on bread characteristics (Le Lay et al., 2016). Although the dominance of LAB species in sourdough has been shown, Ventimiglia et al. (2015) concluded that *Lpb. plantarum* usually manifests as co-dominant with heterofermentative LAB. Another species, *Lcb. casei*, is found in the microbiota of sourdough baked goods and has been employed in sourdough medium in several studies (García-Mantrana et al., 2016; Kitahara et al., 2005) Additionally, some studies have shown that this species can produce exopolysaccharides and has been verified as part of sourdough starter culture (Galle et al., 2011).

According to Ajith and Sunita (2017) LAB-treated bread did not show any fungal contamination for up to four days. This observation aligns with the shorter shelf life reported for some LAB strains, such as *Lactobacillus amylovorus* and *Liml. reuteri*, as indicated in Table 4, depending on the strain and specific application (Ajith and Sunita, 2017). The primary metabolites of LAB fermentation, including acetic/lactic acid, also hinder the proliferation of *Rhizopus* sp. and *Mucor* sp. to 40 and 20%, respectively. Di Biase et al. (2014) and Schieber and Saldaña (2009) observed *A. niger* growth after seven days of baking was slower in LAB-inoculated bread than in control bread. Axel et al. (2015) tested *L. amylovorus* DSM 19280 as a sourdough starter culture for antifungal activity (Axel et al., 2015). Instead of exhibiting mold after 2 days with control samples, it increased the shelf life of bread by an additional four days. In another study, Cizeikiene et al. (2013) tested *Pediococcus acidilactici* strains KTU05-10, KTU05-7, and KTU05-8 in sourdough. Adding sourdough generated with these strains to bread reduced fungal degradation and development throughout the storage period of 8 days, whereas the control group showed conspicuous fungal colonies (Cizeikiene et al., 2013). Additionally, *Lpb. plantarum* LB1 and *Furfurilactobacillus rossiae* LB5 were added to *Penicillium roqueforti* DPPMAF1 to determine how well they killed the fungi. After 21 days from

inoculation, the wheat germ bread sample showed mycelial growth with only 10% contamination (Bullerman and Bianchini, 2007).

Lentilactobacillus diolivorans and *Lentilactobacillus buchneri*, two active propionate producers, were applied to mold-damaged bread to inhibit fungal growth and improve shelf life. Zhang et al. (2010) demonstrated that these strains effectively suppressed mold growth for over 12 days (Zhang et al., 2010). In addition, Ran et al. (2022) found that a mixed culture of *Propionibacterium freudenreichii* D6 and *Lpb. plantarum* L9 significantly delayed the growth of *A. niger* in bakery systems, with acetic acid being the primary contributor to antifungal activity (Ran et al., 2022). Interestingly, *in situ* spraying of bakery products with selected strains of *Liml. reuteri* 5,529, *Levilactobacillus spicheri* O15, and *Leuconostoc citreum* L123 delayed fungal growth when sprayed directly onto pound cake and milk bread rolls contaminated with spoilage fungi, showing strong in situ antifungal activity.

A recent study identified three new LAB strains (*Lpb. plantarum* jQ 301,799, *Lcb. casei* jQ 412,732, and *Levil. brevis* IBRC-M10790) isolated from Tarhana (Tafvizi and Tajabadi Ebrahimi, 2015) in sourdough. The study evaluated the bacterial attributes of these strains and their impact on the texture and preservation of toast over a six-day storage period, showing the production of diverse metabolites by LAB. Additionally, this study aimed to evaluate the potential synergistic or antagonistic effects of LAB combined in sourdough. Settanni et al. (2011) identified *Lpb. plantarum* and *Levil. brevis* among the predominant LAB strains during tarhana fermentation, showing how controlled fermentation parameters influence microbial diversity and support their potential use in bio-preservation (Settanni et al., 2011). Hadaegh et al. (2017) explored the effect of sourdough infused with three newly identified individual LAB strains (*Levil. brevis* IBRC-M10790, *Lpb. plantarum* jQ 301,799, and *Lcb casei* jQ 412,732), and mixed strains on the qualitative attributes of toast bread. The study assessed parameters such as microbial preservation, texture, and bread staling. While the sourdough concentration significantly enhanced microbial preservation by inhibiting spoilage microorganisms, it had minimal effect on the decrease in enthalpy, which reflects the thermal stability or structural integrity of the bread during storage. Mixed LAB strains produced the highest quantity of organic acids, lowering the enthalpy and hardness of bread and improving microbial preservation. Among single strains, *Lcb. casei* reduced bread hardness, improved bread volume, and had the best staling rate (Hadaegh et al., 2017).

LAB antifungal potential in dairy foods

The antifungal activity of LAB has been demonstrated in a number of fermented dairy products, such as cheese and yogurt (Garnier et al., 2017). In addition, LAB strains from species such as *Lpb. plantarum*, *Lcb casei,* and *Lcb. rhamnosus* have shown antifungal effects, which could result in extended shelf-life of dairy products (Leyva Salas et al., 2017). Souza et al. (2023) showed that various LAB strains isolated from cheese and dairy settings hinder the growth of *A. niger* IOC 207 and *Penicillium chrysogenum* IOC 132 (Souza et al., 2023). This suggests that they may be used as biocontrol agents to expand the shelf life of cheese as an alternative to chemical preservatives or thermal processing. Leyva Salas et al. (2018) conducted a study testing two combinations of LAB strains, A1 and A3, for their antifungal effects on dairy products such as sour cream and semi-hard cheese. Both combinations included *Lpb. plantarum* L244, paired with either *Schleiferilactobacillus harbinensis* L172 (A1) or *Lcb. rhamnosus* CIRM-BIA1113 (A3). The A1 combination notably delayed fungal growth in sour cream for up to 24 days and in semi-hard cheese for up to 6 days (Leyva Salas et al., 2018). In addition, Cosentino et al. (2018) concluded that a combination of four Lactobacilli strains could control mold on caciotta cheese without affecting the taste, suggesting the potential of LABs in prolonging the shelf life of dairy (Cosentino et al., 2018).

The antifungal properties of LAB are linked to their ability to generate metabolites, such as fatty acids, organic acids, and proteins. Notably, *in situ* production of these antifungal metabolites by LAB cultures yielded concentrations significantly lower than the minimum inhibitory concentrations (MICs). Importantly, this means that LAB metabolites may interact synergistically with each other (Cosentino et al., 2018). The ability of LAB to deplete manganese (Mn) presents a novel strategy for slowing the proliferation of fungal spoilage in dairy foods, offering the dairy industry a non-destructive means of controlling fungal contamination. Supporting this mechanism, *Lcb.*

TABLE 4 Studies showing the use of LAB species and strains to improve the shelf life of bread.

Starter culture or strain	Shelf-life extension	References
Lpb. plantarum 1A7	Up to 28 days	Coda et al. (2011)
Lpb. plantarum	>14 days	Coda et al. (2011), Gardner et al. (2002), Gerez et al. (2015), Mo and Sung (2014)
Pedioc. acidilactici KTU05-7, *Pedioc. pentosaceus* KTU05-8, KTU05-10	8 days	Cizeikiene et al. (2013)
Propionibacterium freudenreichii, Lpb. plantarum	7 days	Ran et al. (2022)
Lpb. plantarum	4 days	Axel et al. (2015), Cizeikiene et al. (2013); Sadeghi et al. (2019)
Levil. hammesii	6 days	Black et al. (2013), Maldonado et al. (2015), Schieber and Saldaña (2009), Wei et al. (2009)
L. amylovorus	4 days	Axel et al. (2015)
Liml reuteri, Levil. brevis	2 days	Axel et al. (2015), Cizeikiene et al. (2013), Ribes et al. (2018), Zhang et al. (2010)

Pedioc, Pediococcus.

rhamnosus LRH01 and *Lpb. plantarum* LP01 have been shown to inhibit the growth of *Penicillium* strains commonly found in dairy products, both in laboratory media and yogurt serum. Notably, when Mn was reintroduced into the yogurt matrix, the antifungal effect was reduced or completely lost, indicating that manganese depletion is essential for inhibiting *Penicillium* growth (Shi and Maktabdar, 2022). Predicting mold sensitivity and the success of bioprotective cultures in food preservation requires consideration of multiple factors, including the composition of the food matrix, such as Mn levels, and storage conditions (Shi and Maktabdar, 2022). Understanding the complex interactions between LAB and spoilage fungi across various environments is required to optimize inhibitory strategies.

Antifungal activity of LAB in fruit and vegetable products

LAB are key contributors to the protection of fruits and vegetables against fungal attack and mycotoxin contamination. Chen et al. (2021) isolated 13 strains with fungicidal activity out of 224 LAB strains from cured pickles. Among the tested strains, *Lpb. plantarum* CWXP24 and *Lpb. plantarum* CKXP13 showed the highest efficacy against *Penicillium digitatum* on citrus fruits, inhibiting decay and reducing lesions (Chen et al., 2021). *Lcb. casei* YZU01 broke down patulin in fresh apple and pear juices within 36 h of incubation, showing promise for toxin removal in commercial juice products.

Leuconostoc mesenteroides subsp. *mesenteroides* (LB7), grown from apple skin, has not only antifungal activity but also reduces the levels of patulin, although with variable efficiency depending on the type and juice contamination level. The factors determining the efficacy of LAB include pH (5.5–7.0 for optimal antifungal production), bacterial concentration, nutrient availability (glucose at 1.5% enhances production in certain media), competitive bacteria, and incubation conditions (temperature and duration). These conditions not only influence the growth of LAB but also the yield and activity of their antifungal substances (Dalié et al., 2010).

LAB strains are used not only for reducing microbial contamination on produce but also for extending its shelf life. While applications of LAB are currently under consideration for food systems, close scrutiny is necessary, as they may affect product texture, flavor, and many other characteristics (Chen et al., 2021). Some studies have indicated that LAB strains such as *Lpb. plantarum* LO3 and *Pediococcus pentosaceus* can impede mold development in different food systems without affecting taste or other physicochemical criteria, which eventually prolongs the storage period (Crowley et al., 2013).

Food preservation techniques can utilize bacterial-enriched edible coatings containing *Lpb. plantarum* to extend the shelf life of fresh produce. These coatings inhibit fungal growth through the production of organic acids. In some strains, such as *Lpb. plantarum*, bacteriocin production may also contribute to antimicrobial activity, creating unfavorable conditions for spoilage microorganisms. Additionally, they help to preserve the physicochemical properties of fruits, such as texture and moisture content, during storage. This approach provides a natural and effective method for enhancing the safety and longevity of perishable goods, reducing spoilage and waste (Agriopoulou et al., 2020a).

Antifungal activity of LAB in meat products

Undesirable mold control is one of the top priorities for the meat sector, particularly for processed meat. Controlling these undesirable microorganisms will not only ensure the production of safe products but also extend their shelf life while preserving their organoleptic and sensory attributes. In recent years, numerous studies have investigated the antifungal properties of LAB in various processed meat products. Processed meat is a good source of protective LAB in the species such as *Lactococcus lactic*, *Leuconostoc mesenteroides*, *Pediococcus acidilactici*, *Lpb. plantarum*, and *Carnobacterium maltaromaticum*. These LAB strains exhibit antifungal activity through the production of a wide range of antifungal compounds, such as organic acids (e.g., lactic acid), cyclic peptides, reuterin, and other low-molecular-weight bioactive metabolites. While some of these mechanisms have been discussed earlier, they are particularly relevant in meat products, where high moisture and protein levels create an ideal environment for fungal growth. In these systems, the ability of LAB to modify environmental conditions, by lowering pH and reducing water activity, plays a key role in controlling spoilage. For example, LAB can delay mold spoilage in fermented sausages by producing antifungal metabolites, including phenolic acids (such as phenyllactic and benzoic acid) and volatile compounds (such as phenylethyl alcohol and nonanoic acid), which remain active during storage (Nazareth et al., 2023).

LAB have been shown to be effective in managing spoilage and pathogenic fungi in a variety of processed meat products. Álvarez et al. (2020) showed that *Enterococcus faecium* isolated from aged sausage significantly decreased the growth of *Penicillium verrucosum* and *Penicillium nordicum* and its production of mycotoxins under parameters that mimic the ripening process of dry-fermented sausage (Álvarez et al., 2020). *Staphylococcus xylosus*, isolated from the surface of jamón ibérico, inhibited *Penicillium nordicum* growth in dry-cured ham-based medium and reduced ochratoxin A production. An examination led by Zdolec et al. (2008) showed the safety and quality benefits of fermented sausages from Croatia when enhanced with *Latilactobacillus sakei* and mesenterocin. They noted a significant reduction of yeasts without altering the sensory characteristics of the sausage (Zdolec et al., 2008). Najjari et al. (2020) have shown that a mixture of *Latil. sakei* 23 K, *Latil. sakei* BMG95 and *S. xylosus* SUB7037332 XYLO MT111928 was effective for controlling yeast and molds in dry-fermented sausages in Tunisia (Najjari et al., 2020). Significant inhibition of yeast and mold was achieved in vacuum-packaged sliced beef by a combination of *Latil. sakei* CECT4808, and *Latilactobacillus curvatus* CECT 904 (Katikou et al., 2005). The combination of strains can enhance antifungal efficacy through synergistic effects while also improving product quality and shelf life and reduce the risk of resistance in spoilage fungi.

Conclusion and future directions

The food industry faces ongoing challenges in preserving the quality and safety of perishable food items, while simultaneously prolonging their shelf life. Foods naturally contain microbial communities whose composition is dependent on the nature, origin, and handling or storage conditions of the food. The diversity and complexity of the food microbiome pose significant challenges to the

food industry. While food spoilage has been studied for decades, certain microorganisms, such as fungi, particularly those thriving in complex environments or contributing to secondary spoilage through toxin production, were previously underexplored or underestimated in their impact on food safety and quality. Moreover, these microorganisms can evade the traditional preservation methods which primarily target bacterial contamination. However, they are now recognized for their significant role in the rapid spoilage of certain foods. Consequently, the development of new strategies to control fungal contaminants is imperative in the food industry. This urgency is amplified by the increasing consumer demand for natural and safe foods with longer shelf life, as well as the need to reduce food waste and promote sustainable practices in food production and storage.

Studies on the potential of LAB to prevent fungal growth and offsetting mycotoxins have accelerated in the last two decades. However, the antifungal and mycotoxin binding/detoxification properties of LAB isolated from various sources require further investigation.

Discovering LAB strains capable of restraining the growth of mycotoxigenic fungi, particularly those already adapted to specific products, optimizing processing conditions for maximum inhibitory effect, and understanding the molecular mechanisms underlying such inhibition will not only enhance our capacity to produce safer and higher-quality food and feed but also extend their shelf life. Therefore, this will help to reduce significant annual losses in the food industry caused by fungal infestation, contamination, and the presence of mycotoxins. These isolates facilitate multi-step methods for reducing the presence of fungi in the food supply chain. Although no individual isolate or strain can comprehensively address all fungal or food-related challenges, selecting LAB strains that are naturally adapted to the product environment may improve their efficacy. Incorporating such LAB isolates, already recognized as part of the natural microflora in some products, can enhance shelf life, stability, and yield value-added products with a high consumer acceptance rate. However, the success of this new approach remains dependent on several key research and development steps. Future work in this promising field should focus on isolating and characterizing new food-adapted protective cultures with antifungal activity, conducting in-depth structural and functional analyses of antifungal metabolites, and elucidating their mechanisms of action and spectrum of antifungal activity. Additionally, efforts should be directed toward developing eco-friendly and economically viable industrial processes for the large-scale production of novel antifungal ingredients containing protective cultures and/or their active compounds. Finally, large-scale proof-of-concept studies should be conducted to provide more robust scientific data on the effectiveness of these active antifungal ingredients.

Author contributions

MR: Conceptualization, Validation, Visualization, Writing – original draft, Writing – review & editing. SS: Conceptualization, Validation, Visualization, Writing – original draft, Writing – review & editing. GL: Writing – review & editing. SK: Writing – review & editing. IF: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

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ARTICLES FOR FACULTY MEMBERS

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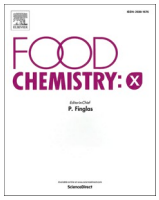

Recent advances of fermented fruits: A review on strains, fermentation strategies, and functional activities

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ABSTRACT

Fruits are recognized as healthy foods with abundant nutritional content. However, due to their high content of sugar and water, they are easily contaminated by microorganisms leading to spoilage. Probiotic fermentation is an effective method to prevent fruit spoilage. In addition, during fermentation, the probiotics can react with the nutrients in fruits to produce new derived compounds, giving the fruit specific flavor, enhanced color, active ingredients, and nutritional values. Noteworthy, the choice of fermentation strains and strategies has a significant impact on the quality of fermented fruits. Thus, this review provides comprehensive information on the fermentation strains (especially yeast, lactic acid bacteria, and acetic acid bacteria), fermentation strategies (natural or inoculation fermentation, mono- or mixed-strain inoculation fermentation, and liquid- or solid-state fermentation), and the effect of fermentation on the shelf life, flavor, color, functional components, and physiological activities of fruits. This review will provide a theoretical guidance for the production of fermented fruits.

1. Introduction

Fruits serve as crucial sources of diverse nutrients, such as dietary fiber, vitamins, and polyphenols, contributing to their status as a healthful food option due to their low fat and calorie content. Studies have shown that regular consumption of fruit could mitigate the risk of various diseases including osteoporosis, diabetes, liver dysfunction, metabolic syndrome, and atherosclerosis caused by poor lifestyle habits (Hasegawa, Kawasaki, Ogawa, Sugiura, & Yano, 2023). However, the richness of nutrients and high moisture content in fruits make them conducive to the proliferation of various microorganisms, particularly parasitic and saprophytic fungi. Fruit deterioration encompasses both pre-harvest and post-harvest stages, with post-harvest microbiological contamination emerging as the main cause of fruit deterioration. At the post-harvest stage, the nutrients in the fruit provide a favorable environment for the growth of microorganisms, particularly yeasts and molds (Zhao, Ndayambaje, Liu, & Xia, 2022). This susceptibility to spoilage not only leads to substantial economic losses but also gives rise to the production of toxins, posing a significant threat to consumer health. For example, patulin, a prevalent fruit toxin produced by *Penicillium* and *Aspergillus*, has been identified for its potential to inflict harm to several organs, including the kidneys (Hou et al., 2022) and liver (Zhang et al., 2022). Hence, the exploration of strategies to mitigate fruit spoilage is of utmost importance.

Proper fruit processing is a good solution to the problem of perishable fruit. However, conventional methods like quick-freezing and blanching have been found to significantly diminish the nutritional content of fruits. In recent years, probiotic fermentation has become an effective way to counteract fruit spoilage due to it can not only extend the shelf life of fruits by inhibiting the proliferation of harmful bacteria but also enhances the flavor and preserves the beneficial substances (Muhialdin, Kadum, Zarei, & Hussin, 2020; Sevindik et al., 2022; Wang et al., 2023). Notably, fermentation stands out as a cost-effective and energy-efficient process.

Fermented fruits are produced by the intricate interaction of microorganisms with the natural fruit medium rich in glucose and fructose. This process facilitates easy storage and transportation, and has witnessed a recent surge in development. Recently, various beneficial microorganisms, such as yeast (Velenosi et al., 2021), *Lactobacillus* (Meng et al., 2022), and *Acetobacter* (Paz-Arteaga et al., 2023), have played a pivotal role in the rapid evolution of fermented products derived from fresh fruits and their by-products. Fermentation contributes greatly to

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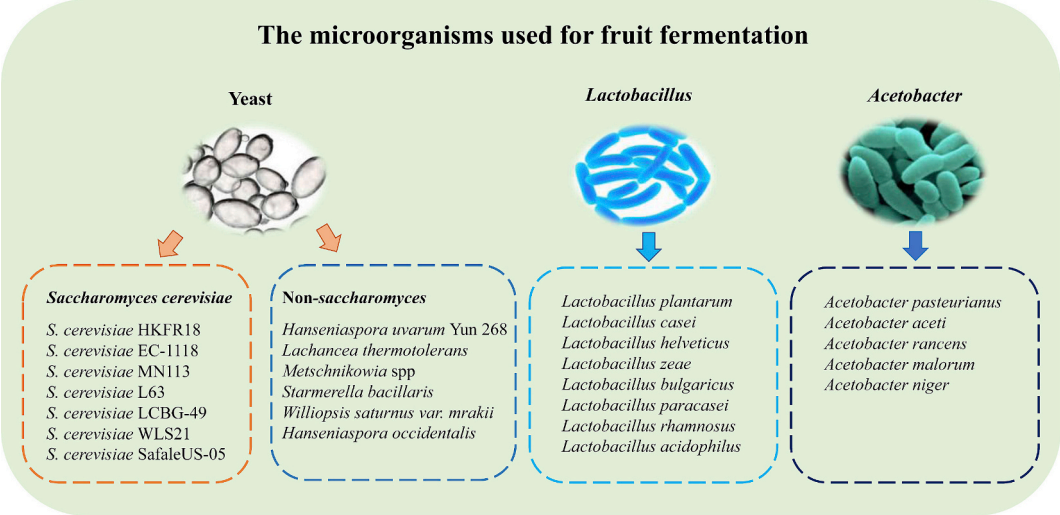

Fig. 1. The strains used for fruit fermentation including *Saccharomyces cerevisiae*, non-*saccharomyces*, *Lactobacillus,* and *Acetobacter*.

the functional activity of the fruits. On the one hand, microorganisms in fermented products could regulate gut microbiota and produce beneficial microbial metabolites such as organic acids (e.g., short-chain fatty acid and gamma-aminobutyric acid) (Ma et al., 2021). On the other hand, fermentation can increase the content of functional nutrients of fruits including polyphenols, flavonoids, organic acids, polysaccharides, amino acids, vitamins, minerals, and other efficacious components, giving the fruit excellent antioxidant, antibacterial, anti-inflammatory, and gut microbiota modulation activities (Feng, Wu, & Weng, 2022; Sheng et al., 2021). Moreover, microbial fermentation can impart distinctive fruity and floral aromas to fruits through the production of esters, ketones, alcohols, terpenes, etc. (Sevindik et al., 2022).

The choice of strains and fermentation strategies emerges as crucial determinants impacting the overall quality of fermented products. These factors play a pivotal role not only in ensuring fermentation uniformity but also in influencing the safety, flavors, and nutritional profiles of the end products. In order to provide a theoretical reference to the development of fermented fruits, this review summarized the commonly used microorganisms and their fermentation strategies in fruit fermentation. Additionally, the review delves into the multifaceted effects of microbial fermentation on the nutritional value, flavor, color, and shelf life of fermented fruit products.

2. Strains used for fruit fermentation

Fruit fermentation refers to the fermentation of one or more fresh fruits under the action of single or composite strains for a certain period. Typically, strains such as lactic acid bacteria, acetic acid bacteria, and yeasts are employed in fruit fermentation, which play a pivotal role in converting sugars present in fruits into alcohol, acetic acid, lactic acid, and other bioactive components, including polyphenols and bioactive peptides, endowing the fruits with distinctive flavor and nutritional values (Fig. 1).

2.1. Yeast

Yeasts are a group of unicellular eukaryotic organisms, which are widely used in the production of fruit wines, fruit enzymes, jams, and more. Throughout the fermentation process, yeasts play a pivotal role in converting the glucose and fructose present in fruits into alcohol and carbon dioxide. Simultaneously, vitamins, amino acids, and other beneficial metabolites were produced. *Saccharomyces cerevisiae* are the most used strains in fruit wine fermentation. Currently, the specialized commercial strains of *Saccharomyces cerevisiae* employed in fruit wine fermentation mainly include *Saccharomyces cerevisiae* HKFR18 (Baek, Kim, Park, Son Jong, & Shim Jae, 2021), *Saccharomyces cerevisiae* EC-1118 (Jiang, Lu, & Liu, 2020), *Saccharomyces cerevisiae* MN113 (Francesca et al., 2023), *Saccharomyces cerevisiae* L63 (Andrade Koelher, de Souza, da Costa, & Aguiar-Oliveira, 2022), *Saccharomyces cerevisiae* LCBG-49 (Edward-Rajanayagam et al., 2023), *Saccharomyces cerevisiae* WLS21 (Li et al., 2022), *Saccharomyces cerevisiae* SafaleUS-05 (Cioch-Skoneczny, Krolak, Tworzydlo, Satora, & Skoneczny, 2023), etc. In recent years, to overcome the drawback of single and convergence flavor caused by commercial *Saccharomyces cerevisiae*, there has been a growing trend to isolate and cultivate more unique *Saccharomyces cerevisiae* from typical fermented foodstuffs using natural and industrial screening methods. Moreover, to generate more aroma compounds, non-*saccharomyces* received increasing attention, which plays an indispensable role in fruit wine fermentation. Compared to *Saccharomyces cerevisiae*, non-*saccharomyces* exhibit higher extracellular enzyme activity, which could hydrolyze more aroma precursors to release abundant aroma substances, thus giving complex aromas to fruit wines (Renault, Coulon, de Revel, Barbe, & Bely, 2015). Concurrently, certain non-*saccharomyces* can produce more complex metabolites (e.g., esters, higher alcohols, and glycerol) to reduce alcohol content. The synergistic fermentation with non-*saccharomyces* and *Saccharomyces cerevisiae* was increasingly used in fruit wine production, which could produce superior flavor. For example, a study by Hu, Jin, Xu, and Tao (2018) demonstrated that collaborative fermentation with *Hanseniaspora uvarum* Yun 268 and *Saccharomyces cerevisiae* increased the production of medium-chain fatty acid ethyl esters in wines, imparting a unique flavor. Another study conducted by Binati et al. (2020) suggested that sequential inoculation of specifically selected strains (*Lachancea thermotolerans, Metschnikowia* spp. and *Starmerella bacillaris*) and *Saccharomyces cerevisiae* EC 1118 positively modulated some relevant chemical parameters and improved the aromatic intensity of wine by increasing the levels of lactic acid, higher alcohols, esters, and glycerol, while reducing ethanol, acetaldehyde, SO_2, and volatile phenols contents. Beyond flavor enhancement, some beneficial compounds (e.g., melatonin) can be produced by non-*saccharomyces*, improving the nutritional value of the fruit wines (Capece et al., 2018).

2.2. Lactic acid bacteria

Lactic acid bacteria are a group of gram-positive bacteria that can ferment sugars to produce lactic acid, which are widely distributed in nature, mainly in fermented foods (such as yogurt), intestinal tracts of animals, soil, water, etc. Lactic acid bacteria play a crucial role in fruit

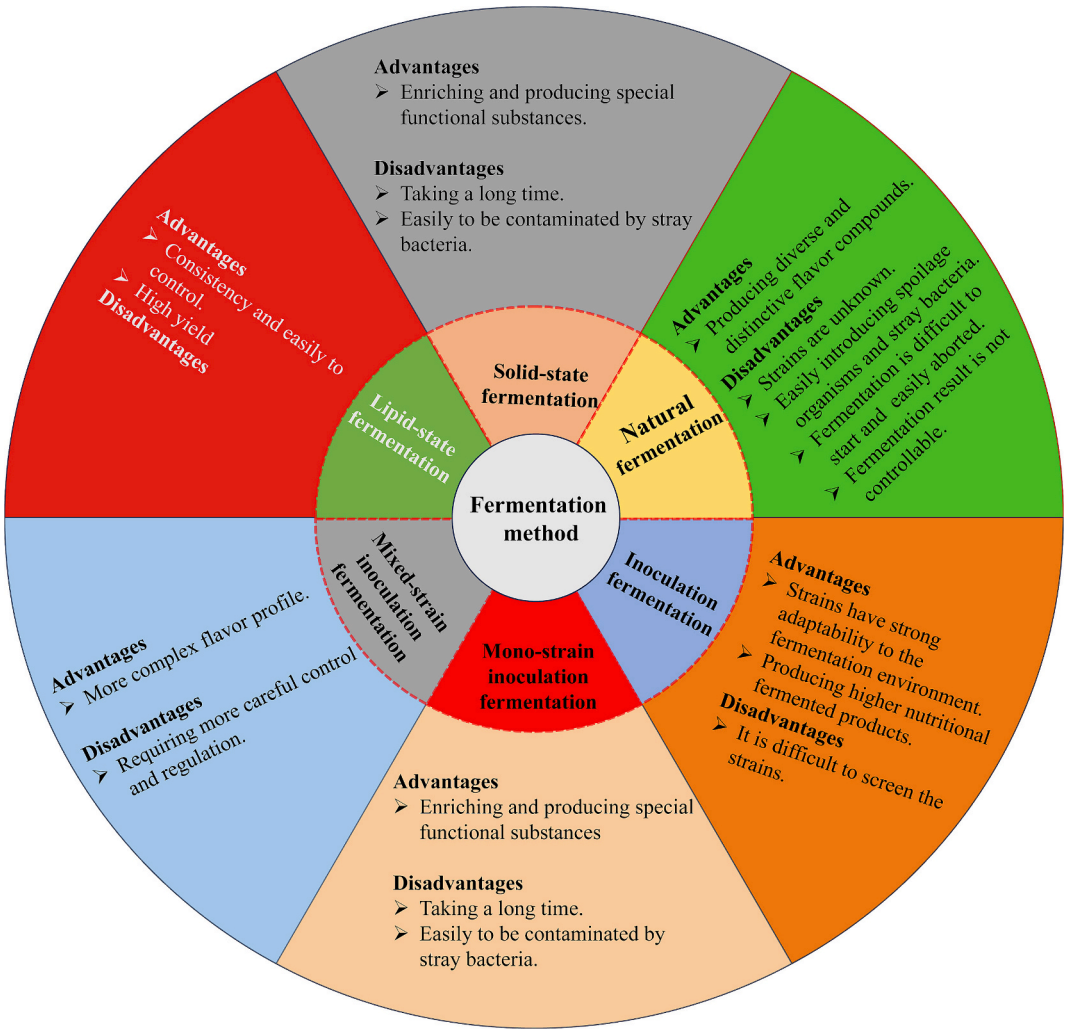

Fig. 2. The fruit fermentation method including natural fermentation, inoculation fermentation, mono-strain inoculation fermentation, mixed-strain inoculation fermentation, lipid-state fermentation, and solid-state fermentation.

fermentation by utilizing carbon sources and free amino acids to produce abundant metabolites including organic acids, bioactive peptides, fatty acids, extracellular polysaccharides, vitamins, etc. The fermentation process of lactic acid bacteria encompasses glycolysis and other alternative pathways. Glycolysis is a process of converting sugar molecules to lactic acid, ethanol, carbon dioxide, etc. Under anaerobic conditions, lactic acid bacteria generate energy through lactic acid fermentation. Simultaneously, lactic acid and other organic acids were produced, which could lower the pH of the environment, discourage the growth of harmful microorganisms, and give fermented fruits special flavor and texture. *Lactobacillus plantarum* (Zhao et al., 2021), *Lactobacillus casei* (Bancalari, Castellone, Bottari, & Gatti, 2020), *Lactobacillus helveticus* (Bancalari et al., 2020), *Lactobacillus zeae* (Inayah, Wibowo, Julianti, & Suciati, 2022), *Lactobacillus bulgaricus* (Oberg, McMahon, Culumber, McAuliffe, & Oberg, 2022), *Lactobacillus paracasei* (Garcia et al., 2018), *Lactobacillus rhamnosus* (Lu, Tan, Chen, & Liu, 2018), and *Lactobacillus acidophilus* (da Silva et al., 2021) are the commonly used *Lactobacilli* in fruit fermentation. Although different *Lactobacilli* play different roles in the fermentation process, their main roles include producing beneficial metabolites such as lactic acid, vitamins, amino acids, polyphenols, etc., lowering the pH value of products to inhibit the growth of harmful microorganisms, prolonging the shelf-life, and improving the texture. For example, a study conducted by Hashemi et al. (2017) showed that the amount of *L. plantarum* LS5 in sweet lemon juice increased from 7.0 ± 0.1 CFU/mL to 8.63 ± 0.38 CFU/mL after 48 h of fermentation, and furthermore, the amount of lactic acid and antioxidant actives (e.g., ascorbic acid) after fermentation also increased significantly, while exhibiting higher antimicrobial properties and antioxidant activity, making it a potential candidate for non-dairy functional beverages. In addition, Kaprasob, Kerdchoechuen, Laohakunjit, and Somboonpanyakul (2018) indicated that cashew apple juice fermented by *L. plantarum*, *L. casei*, and *L. acidophilus* at 30 °C for 48 h increased bioactive substances including concentrated tannins, vitamin C, and phenolic metabolites.

2.3. Acetic acid bacteria

Acetic acid bacteria play a crucial role in the production of fruit vinegar, primarily generating acetic acid and imparting flavor throughout the process. Acetic acid bacteria fermentation involves the oxidation of ethanol to acetic acid and can be categorized into one-step method (simultaneous addition of alcohol and *Acetobacter*) and two-step process (introduction of *Acetobacter* after yeast-mediated alcohol fermentation). Generally, fruit vinegar produced by one-step have better quality such as more diverse flavor, more abundant functional components, and higher fermentation efficiency. Therefore, acetic acid bacteria utilized in fruit vinegar fermentation should have certain ethanol and high-temperature resistance. The screening of high acid-producing acetic acid bacteria with alcohol and temperature tolerance is of great significance in improving the quality of fermented fruit vinegar.

Researchers have striving to isolate high-performing acetic acid bacteria from vinegar grains, vinegar mash, and decay sites of fruits. Recently, *Acetobacter pasteurianus* (Wu et al., 2018), *Acetobacter aceti* (Somboles-tani et al., 2020), *Acetobacter rancens* (Zheng, Liu, Zhang, & Wang, 2010), and *Acetobacter malorum* (Sainz, Mas, & Torija, 2017) stand out as the most frequently employed strains in fruit vinegar fermentation. These *Acetobacter* strains demonstrated the ability to convert alcohol into acetic acid, meanwhile, some volatile aroma components such as organic acids, esters, ketones, and aldehydes were produced, giving fruit vinegar complex aroma and flavor. For example, Xu et al. (2022) showed that coconut water vinegar fermented by active *Acetobacter* exhibited elevated levels of phenyl acetate, isoamyl acetate, and benzaldehyde, imparting an almond, banana, and pear aroma to the coconut water vinegar. In addition, fermented coconut water vinegar contains essential amino acids, especially phenylalanine. Additionally, *Acetobacter* fermentation contributes to the production of antioxidant components such as polyphenols. For instance, pineapple beer vinegar fermented by *Saccharomyces cerevisiae* (LAS01) and *Acetobacter* sp. (ASV03) exhibited higher levels of polyphenols and antioxidant actives (Sossou, Ameya-poh, Karou, & de Souza, 2009). Despite the discovery and application of various *Acetobacter* strains in fruit vinegar fermentation, limited information exists regarding the impact of different *Acetobacter* strains on the quality of the same fruit vinegar. This will be a topic for future research to increase the variety of commercially available fruit vinegars.

2.4. Other strains

Except for the yeast, Lactic acid bacteria, and Acetic acid bacteria, *Leuconostoc mesenteroides* have emerged as a valuable candidate for fruit fermentation. Recently, *Leuconostoc mesenteroides* has been successfully employed in the fermentation of prickly pear. It could enhance the radical-scavenging and antibacterial activities of prickly pear juice (Lee et al., 2016). Moreover, *Leuconostoc mesenteroides* fermentation has the potential to extend the shelf life and improve the rheological, sensory, and functional features of prickly pear fruit puree (Di Cagno et al., 2016). In addition, some *Aspergillus,* especially *Aspergillus oryzae* (Khandelwal, Srivastava, & Bisaria, 2023), *Aspergillus niger* (Saad et al., 2023), and *Aspergillus kawachii* (Miyamoto et al., 2020) were used to ferment the byproducts of fruit, including peel, pomace, and stone using the solid-state method. This approach yields a diverse array of enzymes (e.g., amylase, polygalacturonase, and pectinase) and organic acids (e.g., citric acid, gluconic acid, and gallic acid).

Noteablely, vegetables and fruits contain many common nutrients such as vitamins, minerals, dietary fiber, etc. Differently, vegetables usually contain more dietary fiber and minerals, while fruits contain more sugars and vitamins. Thus, vegetables are more used to make pickled cabbage, salted vegetables, and fermented vegetable juice. The strains suitable for fruit fermentation can also be used for vegetable fermentation, especially lactic acid bacteria and yeast.

Altogether, microorganisms significantly affect the quality of fermented fruits. Nevertheless, the quality of fruits fermented by microorganisms is not only related to fermentation strains but also influenced by fermentation parameters, especially fermentation strategies.

3. Fruit fermentation strategies

According to different division forms, the common methods used for fruit fermentation mainly divided into natural and inoculation fermentation as well as solid- and liquid-state fermentation. Different fermentation methods not only have different applicability but also have a great impact on the quality of the fermented product (Fig. 2).

3.1. The form of fermentation

3.1.1. Natural fermentation

Natural fermentation is a process of using microorganisms from natural environment. Yeast is the first microorganism discovered during the natural fermentation process of bread. Subsequently, *Lactobacillus* and *Acetobacter* were gradually discovered in yogurt and kimchi. With the development of food industry, natural fermentation has been used to produce kiwi wine, persimmon fruit vinegar, and fermented strawberry juice. Generally, compared with inoculation fermentation, natural fermentation can yield a more diverse and distinctive array of flavor compounds. For example, the levels of carboxylic acid, aldehydes, ketones, and phenolic aroma components of natural fermented persimmon vinegar were higher than those by inoculated fermentation (Lu, Zheng, Zhao, & Bai, 2009). Although natural fermentation is a widely used traditional technique, it still has many drawbacks that limit its development as follows: 1) fermentation strains are unknown; 2) spoilage organisms and stray bacteria are easily introduced; 3) fermentation is difficult to start; 4) fermentation is easily aborted; 5) fermentation result is not controllable; 6) heterohydric alcohols, highly volatile acids, and other hazardous substances are easily produced.

3.1.2. Inoculation fermentation

Inoculation fermentation is a process that introduces specific microbial strains into products to be fermented, which presents several advantages over natural fermentation, such as better control of fermentation time, rate, and quality, consistency and predictability of the product, and the avoidance of harmful microorganisms.

Microorganisms suitable for inoculation fermentation include endogenous and commercial strains. Endogenous strains refer to the microorganisms isolated from raw materials, offering distinct advantages such as 1) strong adaptability to the fermentation environment; 2) higher nutritional value of their fermented juice. However, the process of screening specific endogenous strains suitable for fermentation is more complicated and demands a longer experimental period. Yim and Boong (2015) screened acid-, alcohol- and sulfide-resistant *Bartonella* spp. SCMA5 and SCMA6 from traditional fermented foods with the ability to produce large amounts of acetic acid. These strains were found to enhance the antioxidant and hypoglycemic activities of fermented mulberry fruit vinegar. Kanklai, Somwong, Rungsirivanich, and Thongwai (2021) isolated a γ-aminobutyric acid-producing strain, named *Levilactobacillus brevis* F064A, from Thai fermented sausages, and used it to ferment mulberry juice, which significantly increased the contents of antioxidants such as γ-aminobutyric acid and anthocyanins. Ueda et al. (2016) isolated an *L. plantarum* BFRI 380–7 from persimmons and employed it for the fermentation of persimmon syrup, producing a fermented lactic acid drink without milk components.

Commercial strains are specific colonies of microorganisms commonly used to accelerate, control, and improve the fermentation process of beverages and other products. These strains undergo careful screening, cultivation, and propagation to ensure consistent activity and efficacy in specific environments. Currently, the primary commercial strains used for fruit fermentation include *Lactobacillus* such as *L. plantarum*, *L. casei*, *L. acidophilus*, and *Lactobacillus suis*, yeasts such as yeasts UNICAMP-V1, yeasts QA23, yeasts Elegance MP061, yeasts M05 Mead, and *Acetobacters* such as *A. aceti*, *Acetobacter schutzenbachii*, and *A. pasteurianus*. These commercial strains are commonly used to produce fruit fermentation products through mono-or mixed-strain inoculation fermentation.

3.2. The methods of strain inoculation

3.2.1. Mono-strain inoculation

Inoculation fermentation is further categorized into mono-and mixed-strain inoculation fermentation. The process of inoculating a single pure strain to transform substrate is named mono-strain inoculation fermentation. This approach could significantly reduce fermentation time and is particularly favorable for industrial production. However, products fermented with a mono-strain may be somewhat lacking in flavor depth. Mixed-strain fermentation can compensate for

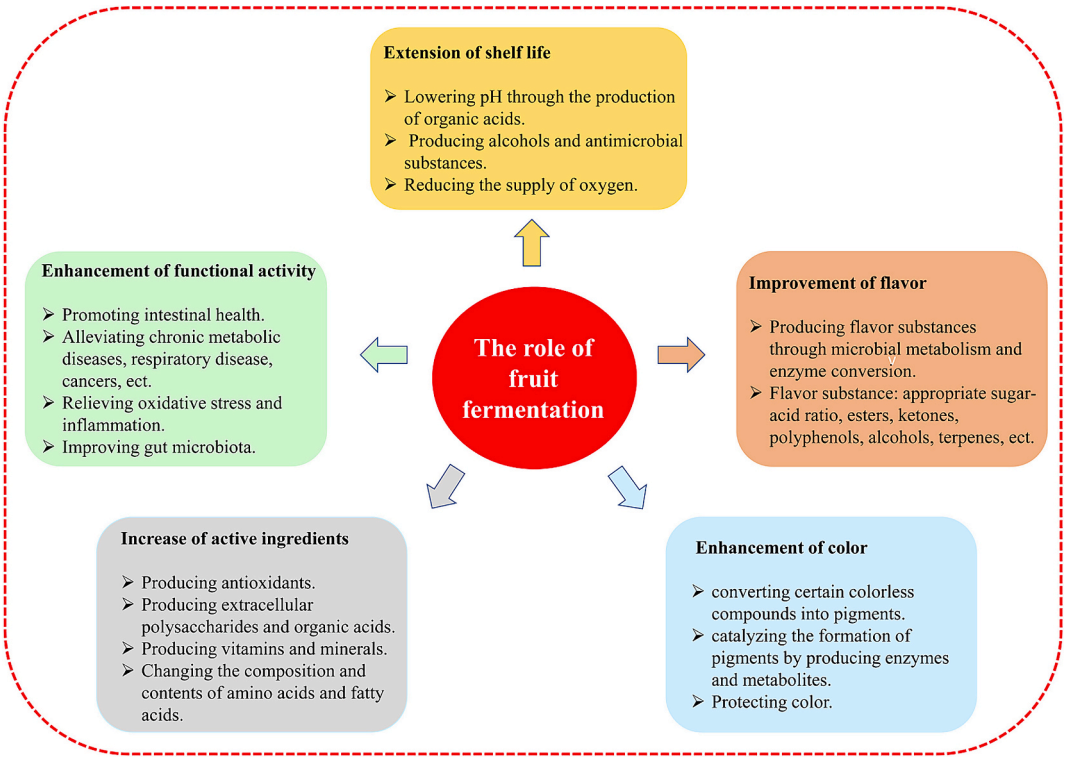

Fig. 3. The beneficial effects of fruit fermentation including the extension of shelf life, the improvement of flavor, the enhancement of color, the increase of active ingredients, and the enhancement of functional activities.

this shortcoming.

3.2.2. *Mixed-strain inoculation*

In mixed-strain fermentation, several different species are utilized to create a more intricate transformation process, leading to the generation of a product with a more complex flavor profile. Li et al. (2017) conducted a study comparing the impact of mono- and mixed-strain inoculation fermentation (utilizing *L. paracasei* 20241, *Bifidobacterium animalis* 6165, *Streptococcus thermophilus* 6063, and *L. acidophilus* 6005) on the quality of apple juice. The results revealed that the contents of alcohols, esters, and other aroma substances were significantly higher than that of mono-strain fermentation, which conferred a stronger fruity and floral flavor. Another study conducted by Sheng et al. (2022) evaluated the effect of mono-and mixed-strain inoculation fermentation with *L. acidophilus* 26 and *L. plantarum* 56 on the quality of red globe grape juice. The results indicated that mixed-strain inoculation fermentation exhibited superior viable bacteria count, total soluble solids, and antioxidant properties compared to mono-strain fermentation. Furthermore, the contents of flavor substances such as lactic acid, acetic acid, ethyl acetate, ethyl benzoate, sorbic acid, and 2-hexenol were significantly increased in the mixed-strain inoculation fermentation. Moreover, when a species produces specific metabolites that contribute to the growth of another species, they may undergo synergistic metabolism (Frey-Klett et al., 2011). It is worth noting that mixed-strain fermentation involves complex interactions of multiple strains and requires more careful control and regulation.

3.3. *The state of fermentation system*

3.3.1. *Liquid-state*

Depending on the state of the medium, fermentation is further categorized into liquid- and solid-state fermentation. Liquid-state fermentation is the primary method employed for large-scale industrial production due to its consistency and ease of control (e.g., temperature, pH, aseptic conditions, and oxygen supply). This method is commonly used in the production of enzymes, fruit wines, and fruit vinegars.

3.3.2. *Solid-state*

Solid-state fermentation is typically applied to ferment the by-products of fruits, such as pineapple pomace, apple pomace, grape pomace, bagasse, citrus rind, pomegranate seeds, mango peel, and banana peel to achieve their high-value utilization. Compared to liquid-state fermentation, solid-state fermentation offers several advantages including high product concentration, low pressure, low energy consumption, and relatively simple equipment. Moreover, solid-state fermentation can enrich and produce some special functional substances. A study has indicated that the enzyme titers in solid-state fermentation are higher than those in deep-liquid fermentation (Roy, Dutta, Sarkar, & Ghosh, 2013). Saeed, Shahid, Naseer, Ghazanfar, and Irfan (2023) achieved the highest fructose yield (7.586 mg/g) through solid-state fermentation of sucrose-rich mango peels using *Bacillus subtilis* at 32 °C, 60% moisture, and pH 7 for 120 h. Aslam et al. (2020) obtained maximum pectinase yield by solid-state fermentation of data palm waste with *Bacillus licheniformis* KIBGE-IB3 at 37 °C and pH 7.0 for 72 h. However, solid-state fermentation has some limitations, as the incubation time is longer than liquid fermentation and the productivity tends to be lower. Moreover, solid-state fermentation is susceptible to contamination by stray bacteria. Despite these limitations, the unique advantages make solid-state fermentation a valuable approach for certain applications, especially in the utilization of fruit by-products.

While various fermentation methods come with distinct targets and associated advantages and disadvantages, they universally contribute to enhancing the sensory characteristics, nutritional value, and storage stability of fruits to a certain extent. Next, the focus of our review will shift to introducing the beneficial effect of fermentation.

4. The beneficial effect of fruit fermentation

Fruit fermentation is a biochemical process where microorganisms

Table 1
Effects of fermentation on shelf life and color of fruits.

Fruits	Strain	Strain source	Fermentation method	Flavor Improvement	Reference
Dragon fruit	*L. plantarum* FBS05	Isolated from fermented traditional Malaysian food	Inoculation fermentation	Enhanced antibacterial activity for dragon fruit juice approximately by three folds; Decreased microbial load and extended shelf life of fresh dragon fruit juice for 3 months at 8 °C after adding 10% fermented dragon juice	(Muhialdin et al., 2020)
Cantaloupe	*L. plantarum* FBS05	Isolated from tempeh (fermented soybean curd)	Inoculation fermentation	Inhibited *E. coli*, *Salmonella typhimurium*, *Aspergillus flavus*, and *Penicillium spp*; Extended the shelf life of fresh cantaloupe juice by 6 months with the addition of 20% fermented cantaloupe juice	(Muhialdin, Kadum, & Hussin, 2021)
Strawberry, grape, and acerola extracts	Traditional water kefir grains	Donated by artisan producers from Maringá, in the state of Paraná, Brazil	Inoculation fermentation	Exhibited antimicrobial potential against *Alicyclobacillus*	(de Menezes et al., 2022)
Sweet lemon juice (*Citrus limetta*)	*L. plantarum* LS5	Obtained from the strain collection of Ferdowsi University of Mashhad, Iran	Inoculation fermentation	Increased antibacterial activity; Against *Salmonella Typhimurium* ATCC 14028 and *E. coli* O157:H7 ATCC 35150	(Hashemi et al., 2017)
Blueberry (*Vaccinium corymbosum* L)	*Bacillus amyloliquefaciens*; *L. brevis*; *Starmerella bombicola*	Isolated from fermented starfish	Mono-strain inoculation fermentation	*Bacillus amyloliquefaciens* and *Starmerella bombicola* enhanced the antimicrobial activity of skin bacteria	(Oh, Jeong, Velmurugan, Park, & Jeong, 2017)
Chagalapoli fruit	*S. cerevisiae* yeast	Purchased from Red Star brand	Mono-strain inoculation fermentation	Increased L^*, indicating the samples are darker; Decreased a^*, indicating a red tone; Increased b^*, obtaining a yellow color	(Flores-Garcia et al., 2019)
Orange (*Citrus sinensis*), tangerine (*Citrus reticulata*), grapefruit (Citrus paradisi)	*Lactic acid bacteria*	Isolated from dairy products (yogurt, cheese, and butter samples)	Mono-strain inoculation fermentation	Increased antibacterial activity, especially for Gram (−) bacteria and fungus; Increased titration acidity, free radical scavenging activity, and total phenolic substance values; Decreased pH, aw, dry matter, viscosity, brix, and L* values	(Akarca & Baytal, 2023)
Apple	*L. acidophilus* BNCC 185342, *L. casei* ATCC 393, *L. plantarum* BNCC 337796	Obtained from Beijing Beina Chuanglian Biotechnology Research Institute (Beijing, China)	Mono-strain inoculation fermentation	Decreased pH; Inhibited *E. coli* and *Staphylococcus aureus*	(Yang et al., 2022)
Mulberry	*Levilactobacillus brevis* F064A	Isolated from Thai fermented sausage	Inoculation fermentation	Inhibited the growth of *Bacillus cereus* TISTR 687, *Salmonella enterica subspecies enterica serovar Typhi* DMST 22842, and *Shigella dysenteriae* DMST 1511	(Kanklai et al., 2021)
Date fruit (*Khastawi*)	*L. plantarum* ATCC 8014	Not mentioned	Inoculation fermentation	Inhibited *Aspergillus niger*, *Aspergillus flavus*, *Escherichia coli*, and *Staphylococcus aureus*; Extended the shelf life of Dodol	(Muhialdin, Kadum, & Hussin, 2021; Muhialdin, Marzlan, et al., 2021)
Strawberry	LAB (*L. plantarum, L. bulgaricus*) and yeast (*S. cerevisiae*)	Obtained from Sichuan Food Fermentation Industry Research and Design Institute, Chengdu City, Sichuan Province, China	Inoculation fermentation	Inhibited *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, and *Bacillus subtilis* ATCC6633; Inhibited the biofilm formation of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 6538	(Zhao, Lan, et al., 2021)
Blueberry	Self-made starters (*L. plantarum*, *L. acidophilus*, *L. paracasei*, *L. rhamnosus*, *L. acidipiscis*, *S. cerevisiae*); Commercial starters (*Bacillus coagulans*, *L. plantarum*, *S. cerevisiae*)	Commercial starters were from Zhejiang Quanzhi Biotechnology Co. Ltd. (Hangzhou, China)	Natural fermentation; Inoculation fermentation of self-made starters and commercial starters	Inhibited the growth of *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella Typhimurium*	(Zhong, Abdullah, & M., Tang, J., Deng, L., & Feng, F., 2021)
Grape	*Oenococcus oeni* MS9 and MS46	Isolated from wine collected from a cellar from Cafayate, Salta, Argentina	Mono-strain inoculation fermentation	Inhibited the activity of *Escherichia coli* 700, *Salmonella Typhimurium*, and *Listeria monocytogenes*	(del Valle, Carmen, Jose, & Maria, 2022)
Mixed fruit juice (pineapple, winter melon, longanhone)	*L. plantarum* TISTR 1465; *L. salivarius* TISTR 1112; *Starmerella boulardii* CNCM I-745	Obtained from the Thailand Institute of Scientific and Technological Research	Mono-or mixed-strain inoculation fermentation	Against *Salmonella Typhi* DMST 22842; Inhibited the formation of Biofilm	(Laosee, Kantachote, Chansuwan, & Sirinupong, 2022)
Grape	*S. cerevisiae* (SCE16 and SCE138); *Starmerella bacillaris* (FA18)	The two *S. cerevisiae* were isolated from 'Savigninin Jura'; *Starmerella bacillaris* was isolated from 'Pinot noir' in Burgundy	Sequential inoculation fermentation	Promoted the evolution of wine color and stabilize it	(Velenosi et al., 2021)

(*continued on next page*)

Table 1 (*continued*)

Fruits	Strain	Strain source	Fermentation method	Flavor Improvement	Reference
Grape	*L. plantarum* Lp39 (CICC6240) and C8–1 (CICC23138); *O. oeni* strains (Viniflora® Oenos and CiNe)	*L. plantarum* Lp39 were received from the China Center of Industrial Culture Collection (Beijing, China); *O. oeni* strains were purchased from Chr. Hansen (Hoersholmm, Denmark)	Mono-strain inoculation fermentation	*L. plantarum* promoted the formation of acetaldehyde during malolactic fermentation; Increased the content of pyranoanthocyanins;	(Wang et al., 2018)
Pineapple residues	*A. niger* GH1 (MUM:23.16)	Obtained from the DIA-UAdeC collection and deposited in the Micoteca of the University of Minho	Solid-state fermentation	Against *Staphylococcus aureus* and *Listeria monocytogenes*	(Paz-Arteaga et al., 2023)

L. brevis, Lactobacillus brevis; *L. acidipiscis, Lactobacillus acidipiscis*; *L. salivarius, Lactobacillus salivarius*; *A. niger, Acetobacter niger.*

(typically bacteria and yeast) transform carbohydrates in fruits into products such as organic acids, alcohols, and gases. This fermentation process plays several essential roles mainly including the extension of shelf-life, the production of flavor and aroma, the enhancement of color, the increase of active ingredients, and the maintenance of health (Fig. 3).

4.1. Extension of shelf life

Fruits, being high in water and nutrients, are prone to microbial attack, especially considering the diverse microorganisms present on their surfaces. In addition, the thin and easily broken skin of some fruits exacerbates the risk of spoilage. Microbial fermentation serves as an effective means to extend the shelf life of fruits. It can inhibit the growth of harmful microorganisms by 1) lowering the pH through the production of organic acids; 2) producing alcohols and some antimicrobial substances such as organic acid, antimicrobial enzyme, and peptide; 3) reducing the supply of oxygen through the production of gases such as carbon dioxide (Table 1). A study conducted by Di Cagno et al. (2008) showed that the use of selected indigenous *Lactobacillus* fermenters extended the shelf life of fermented tomato juice, pineapple fruits, and cherry puree, while maintaining the satisfactory nutritional, rheological, and organoleptic properties of these fruits. Muhialdin et al. (2020) showed that the pH of *L. plantarum* FBS05 fermented dragon fruit juice was reduced from 5.61 to 3.49, which significantly inhibited the growth of *Escherichia coli*, *Salmonella Typhimurium*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. And supplementation of 10% fermented dragon fruit juice extended the shelf life of fresh dragon fruit juice by 3 months. Similar results were obtained in another study conducted by Muhialdin, Kadum, and Hussin (2021). Adding 20% of fermented cantaloupe juice by *L. plantarum* FBS05 extended the shelf life of fresh cantaloupe juice by 6 months when stored at 8 °C. Thus, fermentation is an effective method to extend the shelf life of fruits.

4.2. Improvement of flavor

As the standard of living improves, the pursuit of flavor becomes increasingly passionate. The content and composition of flavor substances in fermented foods play a crucial role in determining their final organoleptic quality and overall product acceptance (Cai et al., 2021). Microbial growth, metabolism, and enzyme-producing capacity are pivotal factors influencing the formation of flavors in fermented foods. In fruits, many aroma precursors such as sugars, glycosides, and amino acids are present, which typically lack distinctive aroma properties. However, through a series of biochemical reactions involving microorganisms and their enzymes (e.g., pectinases, glycosidases, proteases, and lipases), these precursors can be converted into complex aroma compounds, contributing to the development of rich and diverse flavors. In addition, due to the different nutritional components of fruits and the fermentation characteristics of strains employed, the content of organic acids, reducing sugars, and volatile flavor components in the fermented juice is different. In general, sugars, acids, and amino acids serve as the key aromatic compounds in fermented fruit juices, and the appropriate sugar-acid ratio gives the fermented product a moderately sweet and sour taste. Esters, ketones, and phenolics give the fermented fruits sweet and fruity flavor. Alcohols, ketones, and terpenes are associated with the floral and fruity aromas of the fermented fruits. Thus, microorganisms and their metabolic transformation are essential for the flavor presentation of fermented fruit products. Chen, Lu, Yu, Chen, and Tian (2019) found that fermented apple juice produced by *Lactobacillus* contained various flavor substances such as ketones (e.g., 4-heptanone, 2-nonanone, and 4-cyclopentene-1,3-dione), acetaldehyde, esters, and alcohols, improving the final flavor of the product. The study of Di Cagno, Filannino, and Gobbetti (2017) showed that *Lactobacillus* fermentation of pomegranate juice produced well-flavored substances including alcohols, ketones, olefins, and terpenes, while, reduced aldehydes with off-flavors. Ricci et al. (2018) used the strains of *L. plantarum* and *L. rhamnosus* isolated from dairy products and plant substrates for the fermentation of elderberry juice. The results showed that ethyl acetate, methyl isovalerate, isoamyl isovalerate, and methyl salicylate contents increased greatly, which were associated with fruit odor. The specific effects of fermentation on fruit flavor are shown in Table 2.

4.3. Enhancement of color

The color stands out as a pivotal determinant of fermented fruit quality, intricately intertwined with flavor, safety, and nutritional value. Good color has a positive impact on the fermented fruit quality, which can not only enhance its visual appeal but also endow fermented fruits with stronger biological activities by producing natural pigments (e.g., carotenoids, anthocyanins, flavonoids). Raw material pigments, fermentation strain, enzyme, and metabolites produced by microorganisms are important influencing factors for the color formation of fermented fruits. Fruits' inherent pigments precursor serve as substrates for color development, and microorganisms play a transformative role by converting certain colorless compounds into pigments through a series of reactions. For example, colorless flavanols present in grapes and wine are actively involved in oxidative browning, engaging in reactions with anthocyanins to give rise to derived pigments (Lambert et al., 2015). Moreover, enzymes and metabolites produced by fermentation strains can act as catalysts to accelerate the formation of pigments. For example, Acetaldehyde released by *L. plantarum* enhanced the formation of pyran anthocyanins in wine during malolactic fermentation (Wang et al., 2018). In addition, some strains can assist in color retention. For example, various non-*saccharomyces* have a protective effect on wine color, such as *Starmerella bacillis* (Velenosi et al., 2021). Therefore, fermentation is a great method to improve color (Table 1).

4.4. Increase of active ingredients

The widespread appeal of fermented foods can be attributed, in part, to their rich concentration of active ingredients, imparting health

Table 2
Effect of fermentation on the flavor of fruits.

Fruits	Strain	Strain source	Fermentation method	Flavor improvement	Reference
Grape	*S. bayanus* Y4, *Torulaspora delbrueckii* Y7	Isolated from the mash of the fruit wine factory (Chengdu, China)	Mono-or mixed-strain inoculation fermentation	Reduced total acidity and dominant organic acids; Decreased aliphatic compounds; Increased aromatic compounds including acetate esters, ketones, and terpenes	(Liu et al., 2023)
Longan	*W. saturnus* ssp. *saturnus* CBS 254	From CBS Culture Collections (The Netherlands)	Inoculation fermentation	Enhanced the production of isoamyl alcohol and its ester, isoamyl acetate, 2-phenylethanolandits ester, and 2-phenylethylacetate	(Thi-Thanh-Tam, Yu, Curran, & Liu, 2012)
Noni fruit	*Acetobacter* sp. (GDMCC *No.*62221)	Isolated from naturally fermented noni juice	Inoculation fermentation	Decreased or even eliminated hexanoic acid, octanoic acid, and butanoic acid	(Zhang et al., 2023; Zhang et al., 2023; Zhang et al., 2023)
Apricot Juice	*L. plantarum* (LP56)	Obtained from Xiannong Biotechnology (Shanghai) Co. Ltd	Inoculation fermentation	Increased alcohols, aldehyde, acid, and ester, giving fruits pine and citrus flavors	(Sun et al., 2022)
Grewia berries and cantaloupe	*S. cerevisiae* NRRLY-12603, *A. aceti* MCC 2109	*S. cerevisiae* NRRLY-12603 from Culture Collection, Peoria, USA; *A. aceti* MCC 2109 from National Chemical Laboratory, Pune	Sequential inoculation fermentation	Produced ethyl acetate and isopentyl alcohols	(Rudra et al., 2022)
Pomegranate	Autochthonous L. *plantarum* C2 and POM1; Commercial L. *plantarum* LP09	*L. plantarum* C2 and POM1 from Culture Collection of the Department of Soil, Plant and Food Sciences, University of Bari, Italy; *L. plantarum* LP09 from Sacco Srl, Milan, Italy	Sequential inoculation fermentation	Increased desired compounds (e.g., alcohols, ketones, and terpenes); Decreased non-desired aldehydes	(Di Cagno et al., 2017)
Grape	*S. cerevisiae* AWRI838; *Metschnikowia pulcherrima* AWRI3050; *Saccharomyces uvarum* AWRI2846; *Metschnikowia pulcherrima* AWRI3050	*S. cerevisiae* AWRI838, *Metschnikowia pulcherrima* AWRI3050, *Saccharomyces uvarum* AWRI2846 from the Australian Wine Research Institute (AWRI); *Metschnikowia pulcherrima* AWRI3050 from *M. pulcherrima* AWRI1149	Mono-strain or mixed-strain inoculation fermentation	*Metschnikowia pulcherrima* and *S. cerevisiae* AWRI838 showed higher concentrations of ethyl acetate, total esters, total higher alcohols, and total sulfur compounds; *Saccharomyces uvarum* increased the concentration of alcohols	(Varela, Barker, Tran, Borneman, & Curtin, 2017)
European cranberry	Not mentioned	Not mentioned	Natural fermentation	Increased acids, especially 3-methyl-butanoic acid; Increased Ketones and alcohols	(Yilmaztekin & Sislioglu, 2015)
Gilaburu fruit	*L. casei* (Chr. Hansen 431); *L. delbrueckii* subsp. (NBRC3202); *L. plantarum*-23	Obtained from the microbiology laboratory of Adana Alparslan Turkes Science and Technology University located in Turkey	Natural or mono-strain inoculation fermentation	*L. plantarum*-23 increased the contents of phenylethyl alcohol, hexyl acetate, and 3-hydroxy-β-damascone; Provided fruity and floral aroma	(Sevindik et al., 2022)
Mango juice	*W. saturnus var. mrakii* NCYC500, *S. cerevisiae* MERIT ferm	*W. saturnus var. mrakii* NCYC500 from National Collection of Yeast Cultures, Norwich, UK; *S. cerevisiae* MERIT ferm from Chr.-Han., Denmark	Sequential inoculation fermentation	Increased β-citronellol; Improved aroma complexity and balance	(Li, Chan, Yu, Curran, & Liu, 2014)
Italian Riesling grapes	*H. uvarum* YUN268, *P. fermentans* Z9Y-3, and *S. cerevisiae* (Excellence TXL)	*S. cerevisiae* (Excellence TXL) from LAMOTHE ABIET; *H. uvarum* YUN268 and *P. fermentans* Z9Y-3 from the Wine School of Northwest Agriculture and Forestry University	Simultaneous or Sequential inoculation fermentation	Produced more volatile aroma substances, glycerol content, and esters; Enhanced the aroma of lemon, cream, and almond	(Xia, Zhang, Sun, Zhang, & Zhang, 2023)
Grape	*T. delbrueckii*-214 (Accession number: MG017548) and *S. cerevisiae*-1088 (Accession number: MG017577)	Isolated from spontaneous fermentations of Narince grapes	Mono-or mixed-strain inoculation fermentation	Mixed fermentation increased alcohol and ester content; Improved the aromatic intensity and complexity of the wine	(Arslan, Celik, & Cabaroglu, 2018)
Mixed fruit juice (pineapple, winter melon, longan honey)	*L. plantarum* TISTR 1465; *L. salivarius* TISTR 1112; *Starmerella boulardii* CNCM I-745	Obtained from the Thailand Institute of Scientific and Technological Research	Mixed-strain inoculation fermentation	Increased alcohols (3-methyl-1-butanol, 1-hexanol, 2-phenylethanol), acetaldehyde, acetic acid, esters (ethyl acetate, ethyl 2-methylbutyrate, ethyl hexanoate, ethyl lactate, ethyl decanoate), 3-hydroxy-2-butanone, 2,4-di-tert-butylphenol, and linalool	(Laosee et al., 2022)
Sugarcane	*S. cereviasiae*; *S. cereviasiae* CCRC22580	*S. cereviasiae* was isolated from the liquor rice dregs; *S. cereviasiae* CCRC22580 from Bioresource Collection and Research Center (Food Industry and Development Institute, Hsinchu, Taiwan)	Mono-strain inoculation fermentation	Increased 1-methylethyl acetate, isoamyl acetate, ethyl hexanoate, and ethyl octanoate; Enhanced the sweet, fruity, and ester flavor of wines	(Tzeng, Chia, Tai, & Ou, 2010)

(*continued on next page*)

Table 2 (*continued*)

Fruits	Strain	Strain source	Fermentation method	Flavor improvement	Reference
R. roxburghii, blueberry, plum	*S. cerevisiae* (ZYMAFLORE X16), *H. uvarum*	*S. cerevisiae* from LAFFORT (France); *H. uvarum* from China Industrial Strain Conservation Center	Simultaneous or sequential inoculation fermentation	Sequential inoculation positively affected the mellowness of the wine and achieved a better harmony of the overall wine flavors	(Huang et al., 2022)
Chardonnay grape juice added with fruit juices	*S. cerevisiae*	Not mentioned	Inoculation fermentation	Identified 29 major volatile compounds in wine, including 8 alcohols, 12 esters, 6 acids, and 3 miscellaneous compounds	(Patel & Shibamoto, 2003)
Kiwifruit	*L. acidophilus* 85, *L. helveticus* 76, *L. plantarum* 90	Purchased from WECAREBIO company (Jiangsu, China)	Mono-or mixed-strain inoculation fermentation	Improved the formation of total volatile compounds, especially for L. *helveticus* 76	(Wang et al., 2022)
Frozen sea buckthorn named "shengqiuhong"	*L. paracasei*; *S. cerevisiae*	*L. paracasei* from Xi'an Jushengyuan Biotechnology Co., Ltd. (Xi'an, China). *S. cerevisiae* from Angelyeast Inc.	Mixed-strain inoculation fermentation	Increased the sweetness of the sea buckthorn juice; Decreased the fruity flavor; Increased the bitterness	(Wu et al., 2022)
Noni fruits	*L. plantarum* CICC22703	Purchased from the China Center of Industrial Culture Collection	Inoculation fermentation	Increased methyl salicylate content; Increased the percentage of linalool, 1-Hexanol, octanol, 2-Heptanol, and α-Terpineol	(Cheng et al., 2021)
Kiwifruit	*L. plantarum* LG1034; *Pediococcus lactis* LG0259; *Bacillus rhamnosus* LG0262; *L. lactis* LG0827; *L. helveticus* LG4316; *L. paracasei* LG0260; *Kluyveromyces marxianus* J2853; *S. cerevisiae* J2861	Not mentioned	Mono-strain inoculation fermentation	*L. plantarum* LG1034, *Pediococcus. lactis* LG0259, *Bacillus rhamnosus* LG0262, *L. lactis* LG0827, and L. *helveticus* LG4316 increased polysaccharides, γ- aminobutyric acid, organic acids, and volatile compounds	(Cai et al., 2022)
Kiwifruit	*S. cerevisiae* (Drop Acid Yeast, DV10, SY and RW)	*S. cerevisiae* SY and RW from Angel Yeast CO., Ltd. (China). *S. cerevisiae* Drop Acid Yeast and DV10 from Yantai Diboshi CO., Ltd. (China) and Lallemand CO., Ltd. (China), respectively	Mono-strain inoculation fermentation	Increased (E, E)-2,4-heptadienal, fatty flavor, green aroma, 1-octen-3-one, and 4-methyl-2-pentanone fermented by *S. cerevisiae* RW	(Zhang, Chen, et al., 2023; Zhang, Hong, et al., 2023; Zhang, Ma, et al., 2023)
Passion fruit	*L. plantarum* CCMA 0743; *L. paracasei* LBC-81	*L. plantarum* CCMA 0743 from the Culture Collection of Agricultural Microbiology, Federal University of Lavras, Brazil; *L. paracasei* LBC-81 from Danisco, USA	Mono-or mixed-strain inoculation fermentation	Increased octanoic acid and hexyl ester in the mixed-strain fermentation juice	(Fonseca et al., 2022)
Nangao greengage (*Prunus mume*)	Non-saccharomyces yeasts (*Pichia terricola*, *H. occidentalis*, *Candida sorboxylosa*, *Issatchenkia orientalis*); *S. cerevisiae* BV818	Non-Saccharomyces yeasts were selected from spontaneous fermentation of greengage; *S. cerevisiae* BV818 were obtained from Angel Yeast Co. Ltd., Hubei, China	Mixed-strain inoculation fermentation	*Pichia terricola*, *H. occidentalis*, and *Issatchenkia orientalis* degraded citric acid and malic acid; *H. occidentalis* imparted fruity aroma to fermented greengage beverage; *Candida sorboxylosa* provided some higher terpenes with flowery and fruity aroma	(Qiu et al., 2022)
Kiwi fruit	*S. cerevisiae* EC1118 and Jiuqu	Jiuqu from Angel Yeast Co., Ltd. (Yichang, Hubei, China); *S. cerevisiae* EC1118 from Xinmiao Winery (Ya'an, Sichuan, China)	Mono-strain or mixed-strain inoculation fermentation	Mixed fermentation showed high quality of the final products; Decreased total organic acids and methanol contents; Increased lactic acid content	(Chen, Fu, et al., 2019; Chen, Lu, et al., 2019)
Natural 'Langshan' navel orange	*Lactiplantibacillus plantarum* (Lp), *L. fermentum* (Lf), *Lactobacillus acidophilus* (La), *Lacticaseibacillus rhamnosus* (Lr), *Lacticaseibacillus paracasei* (Lc), *Bifidobacterium longum* (Bl),	Lr, Lc and Bl were obtained from the China Center of Industrial Culture Collection (Beijing, China); Lp, Lf, and La were from the Key Laboratory for Fruits and Vegetables storage Processing and Quality Safety in Hunan	Mono-strain inoculation fermentation	Increased aroma-active compounds such as D-limonene, β-caryophyllene, terpinolene, and β-myrcene; Exhibited more desirable aroma flavors such as orange-like, green, woody, and lilac incense after fermentation by Lc	(Quan, Liu, Guo, Ye, & Zhang, 2022)
Apple	*S. cerevisiae* (Sc01, Sc02, Sc05, Sc12, Sc21, Sc24)	Obtained from the Department of Microbiology, HPAU, Palampur, India	Inoculation fermentation	Sc01 fermentation had the highest sensory property	(Kanwar & Keshani., 2016)
Noni fruit	*L. lactis*; *L. cremoris*; *Streptococcus thermophilus*; *L. plantarum*, *Levilactobacills brevis*; *L. acidophilus*, *L. fermentum*; *L. rhamnosus*	*L. rhamnosus* was obtained from Christian Hansen; Others were obtained from the China National Microbial Resource Center (Beijing, China)	Mono-strain inoculation fermentation	Decreased the unpleasant butanoic acid, especially in *L. plantarum* fermented noni fruit juice	(Zhang, Chen, et al., 2023; Zhang, Hong, et al., 2023; Zhang, Ma, et al., 2023)
Pinot Grigio grapes	*Lachancea thermotolerans*; *Metschnikowia* spp.; *Starmerella bacillaris*; *S. cerevisiae* EC 1118	Obtained from Local culture collection of the Department of Biotechnology of the University of Verona	Simultaneous or Sequential inoculation fermentation	*Metschnikowia* spp. promoted the formation of higher alcohols and esters, while reduced volatile phenols; *Starmerella bacillaris* increased glycerol, while reduced acetaldehyde and total SO_2; *Lachancea thermotolerans* increased	(Binati et al., 2020)

(*continued on next page*)

Table 2 (*continued*)

Fruits	Strain	Strain source	Fermentation method	Flavor improvement	Reference
				lactic acid and reduced ethanol content	
Ripe bayberry fruit	*L. plantarum* CGMCC 18099; *Streptococcus thermophilus* CGMCC 18045; *L. acidophilus* CGMCC 18095; *L. bulgaricus* JYLB-19; *L. casei* CGMCC 18096; *L. helveticus* CGMCC 11159	Purchased from Zhongke-Jiayi Biological Engineering Co., Ltd. (Weifang, China)	Inoculation fermentation	Increased the concentrations of characteristic aroma volatiles, such as 2-hexenal, 1-hexanol, and (Z)-3-nonen-1-ol	(Chen et al., 2022)

S. bayanus, Saccharomyces bayanus; *W. saturnus* var. *mrakii, Williopsis saturnus* var. *mrakii*; *W. saturnus* ssp. *Saturnu*, *Williopsis saturnus* ssp. *Saturnu*; *S. cerevisiae, Saccharomyces cerevisiae*; *H. uvarum*, *Hanseniaspora uvarum*; *P. fermentans, Pichia fermentans*; *T. delbrueckii, Torulaspora delbrueckii*; *L. salivarius, Lactobacillus salivarius*; *L. delbrueckii* subsp., *Lactobacillus delbrueckii* subsp.; *H. occidentalis, Hanseniaspora occidentalis*; *L. lactis, Lactobacillus lactis*; *L. fermentum, Limosilactobacillus fermentum.*

benefits to the fruits (Table 3). The diversity in fermentation substrates and strains leads to variations in the types and amounts of these active substances. Generally, fermentation of fruits by *lactobacilli* mainly produces lactic acid, meanwhile, increases the contents of antioxidants (e.g., polyphenols), vitamins (e.g., vitamin C and vitamin K), extracellular polysaccharides, and minerals (e.g., potassium, calcium, magnesium, and iron). A study by Sevindik et al. (2022) showed that natural and inoculated fermentation of gilaburu fruits by three *lactobacilli* of *L. plantarum*, *Lactobacillus delbureckii*, and *L. casei* significantly increased polyphenol contents (e.g., chlorogenic and cryptochlorogenic acids) and minerals-potassium, especially in *L. plantarum* fermented production. Fermenting fresh lychee juice with *L. casei* for 18 h increased extracellular polysaccharide contents to 7.07 g/L, which improved the viscosity of fermented lychee juice (Zheng et al., 2014). In addition, fermentation of fruits by *lactobacilli* could change the composition and content of amino acids and fatty acids. Feng et al. (2022) showed that the levels of γ-aminobutyric acid, L-isoleucine, N-acetylornithine, and taurine in fermented elderberry juice with *L. bulgaricus* BNCC336436 and *Streptococcus thermophilus* ABT-T were significantly increased, while the relative contents of L-asparagine, L-arginine, L-aspartic acid, L-arogenate, and 10-hydroxystearic acid were significantly decreased. In addition, the contents of fatty acids including eicosapentaenoic acid, γ-linolenic acid, and sphingomyelin increased significantly.

S. cerevisiae and non-*saccharomyces* are the key microorganisms in the production of fruit wine. Polyphenols and polysaccharides are the main functional active ingredients in fruit wines, which imparts the fruit wine with excellent antioxidant, anti-inflammatory, and anti-bacterial activities. For example, Liu et al. (2021) isolated polysaccharides with molecular weights greater than 1000 kDa from beef heart plum wine fermented by *Saccharomyces cerevisiae* LalvinEC1118, which possessed great DPPH radical scavenging and α-glucosidase inhibitory activities. Lee et al. (2013) found that total polyphenols and anthocyanin contents in yeast-fermented apple pine wine and apple vanilla wine were higher, which had great antioxidant activities.

Fruit vinegar fermentation inoculated with *Acetobacter* is the next stage of fruit wine fermentation. During this stage, notable changes in bioactive substances occur. In general, the contents of organic acids (e.g., acetic, tartaric, and malic acids) and vitamins increased significantly compared to the alcoholic fermentation stage. For example, compared with black rose fruit wine, a significant increase in organic acids and vitamin C contents was observed in fruit vinegar. The composition and concentration of polyphenols and amino acids in fruit vinegar exhibit variable changes, with increases observed in certain cases and decreases in others. These alterations are contingent upon factors such as the fermenting substrates used, the specific strains involved, and the fermentation conditions applied. For example, the total polyphenol content of black rose fruit vinegar did not change compared with that of black rose fruit wine, while the content of anthocyanins and total flavonoids decreased significantly. Proline and histidine are the main amino acids in Goji fruit wine. During the fermentation of fruit vinegar, histidine increased significantly, while proline and alanine content decreased significantly (Xia et al., 2022). Altogether, fermentation of fruits could produce various active ingredients superior to those of fruit and vegetable raw materials, such as enzymes, organic acids, peptides, oligosaccharides, vitamins, flavonoids, polyphenols, amino acids, natural antibiotics, minerals, polysaccharides, as well as antioxidant components such as γ-aminobutyric acid (GABA), superoxide dismutase (SOD), catalase, etc., to increase its commercial value.

4.5. Maintenance of health

The development of probiotic fermented fruit products not only increases the economic value of fruits, but also organically combines probiotics and their active metabolites including polyphenols, polysaccharides, and dietary fibers. Thus, fermented fruit products exhibit outstanding health-enhancing effects including the promotion of intestinal health, the improvement of oxidative stress and inflammation, and the enhancement of immunity response (Doriya, Kumar, & Thorat, 2022). Furthermore, fermented fruit products have been used to relieve several diseases such as diabetes, cardiovascular diseases, nervous system diseases, liver damage, respiratory disease, and cancers. For example, fermented papaya has been considered a good antioxidant and an excellent nutritional aid for the intervention of Alzheimer's disease, allergic reactions, cancer, and anemia (Leitao, Ribeiro, Garcia, Barreiros, & Correia, 2022). The main reasons for the ability of fermented fruits to alleviate a wide range of diseases include antioxidant, anti-inflammatory, and immunomodulatory activities. Isas et al. (2023) showed that fermented pomegranate juice could ameliorate hyperglycemia, hyperlipidemia, fat deposition, and hepatic tissue damage in high-fat diet-induced obese C57BL/6 mice through its antioxidant activity. Kim et al. (2021) demonstrated that fermented plums of *L. plantarum* and *L. casei* were effective in reducing the risk of cancer via inhibiting oxidative stress and the expressions of pro-inflammatory factors including TNF-α, IL-1β, IL-6, IL-12, and IL-17 in DSS-induced colitis mice.

As the "second brain" of the organism, intestinal microbiota is intricately linked to a variety of chronic metabolic diseases, such as obesity, diabetes, cardiovascular, and cerebrovascular diseases. Fermented fruit products also could improve intestinal microbiota via increasing the abundance of beneficial bacteria and inhibiting the growth of harmful bacteria. A study has indicated that administration with fermented fruit increased the abundance of beneficial bacteria including *Bacteroides*, *Roseburia*, *Butyrivibrio*, *Lactobacillus*, and *Akkermansia*, thus, relieving obesity and hyperlipidemia (Yan, Wang, Weng, & Wu, 2020). While improving the structure of intestinal microbiota, the intestinal micro-environment will be also improved via increasing the levels of short-chain fatty acid and enhancing intestinal immunity (Valero-Cases, Cerda-Bernad, Pastor, & Frutos, 2020). Therefore, fermented fruits are good prebiotics for relieving kinds of diseases.

Although fermented fruits have great benefits, there are still some issues that need to be solved, including 1) The strains that can be used for fermentation is still lacking; 2) The stability of fermented fruit is

Table 3
Effects of fermentation on the active ingredients and functional activities of fruits.

Fruits	Strain	Strain source	Fermentation method	Activities	Reference
Prunus mume juice	*L. plantarum* KCTC 33131; *L. casei* KCTC 13086	Obtained from Korea Collection for Type Cultures (Jeongeup, Korea)	Mixed-strain inoculation fermentation	Alleviated the symptoms of colitis caused by DSS; Inhibited apoptosis of intestinal epithelial cells in DSS-induced colitis mice	(Kim et al., 2021)
Elderberry juice	*L. bulgaricus* BNCC336436; *Streptococcus thermophilus* ABT-T	Obtained from Food Biotechnology Laboratory at Ningbo University, China	Mixed-strain inoculation fermentation	Increased total phenolic, total amino acids, and derivatives; Decreased sucrose, l-fucose, l-malic acid, tartaric acid, and citric acid	(Feng et al., 2022)
Collagen peptide jackfruit juice	Lactic acid bacteria powder (*L. acidophilus*, *L. plantarum*, *Pediococcus pentosaceus*, *L. casei*)	Obtained from Zhongke Jiayi Co. Ltd. (Qingzhou, China)	Mixed-strain inoculated fermentation	Increased anti-oxidant capacity and lactic acid content; Promoted immune organ indexes; Alleviated the injuries of colon tissue; Stimulated cytokines and immunoglobulins; Upregulated TNF-α, IL-4, IFN-γ, IL-2, IL-10, T-bet, Foxp3, ROR-γ, and GATA3 expression; Improved gut microbiota composition and SCFAs concentration	(Ma et al., 2021)
Tremella and blueberry	*L. acidophilus*, *L. rhamnosus*, *L. casei*, *L. plantarum*, *Bifidobacterium longum*, *Bifidobacterium. lactis*, *Bifidobacterium. breve*, *Streptococcus thermophilus*	Obtained from Taiwan sub-core Biotechnology (Taiwan, China)	Mixed-strain inoculated fermentation	Decreased body weight and fat losses; Improved Proteobacteria/ Bacteroidetes ratio; Increased *Allobaculum*, *Blautia*, *Parabacteroides*, and *Prevotella*; Increased short-chain fatty acids	(Sheng et al., 2021)
Blueberry	Self-made starters (*L. plantarum*, *L. acidophilus*, *L. paracasei*, *L. rhamnosus*, *L. acidipiscis*, *S. cerevisiae*); Commercial starters (*Bacillus coagulans*, *L. plantarum*, *S. cerevisiae*)	Commercial starters were from the Zhejiang Quanzhi Biotechnology Co., Ltd. (Hangzhou, China)	Natural fermentation; Inoculation fermentation of self-made starters and commercial starters	Inoculation fermentation is better than natural fermentation; Increased antioxidant potentials; Increased α-glucosidase and α-amylase inhibitory activities; Promoted the glucose consumption of HepG2 cells	(Zhong et al., 2021)
Loquat juice	*L. plantarum* LZ 22, *L. acidophilus* CICC®20709	*L. plantarum* LZ 22 was isolated from the highland barley wine koji in Tibet, China; *L. acidophilus* CICC®20709 was from the China Center of Industrial Culture Collection	Mono-strain inoculation fermentation	Enhanced antioxidant activity; Increased total flavonoid; Differentially regulated metabolites mainly in lipids and lipid-like molecules, organic acids and derivatives, amino acids, peptides, and analogs	(Meng et al., 2022)
Sea buckthorn	*L. plantarum* RM1 (MF817708)	Isolated from the Department of Food Technology, City of Scientific Research, (Rayeb milk)	Inoculation fermentation	Enhanced the functional phenolic and flavonoid contents, antioxidant, and antimicrobial activities; Increased ACE inhibitory and anticancer potentials	(El-Sohaimy et al., 2022)
Acerola and guava fruit by-product	*L. plantarum* 53, *L. paracasei* 106, *L. fermentum* 56, *L. casei* L-26	Not mentioned	Mixed-strain inoculation fermentation	Increased the contents of total flavonoids and total phenolics; Increased antioxidant activity	(de Oliveira et al., 2020)
Mixed fruit juice (pineapple, winter melon, longan honey)	*L. plantarum* TISTR 1465; *L. salivarius* TISTR 1112; *Saccharomyces boulardii* CNCM I-745	Obtained from the Thailand Institute of Scientific and Technological Research	Mono-or mixed-strain inoculation fermentation	Exhibited antioxidant activity, especially the mixed fermentation	(Laosee et al., 2022)
Prickly pear fruits	Not mentioned	Not mentioned	Not mentioned	Increased total phenolic compounds and antioxidant content	(Ben Hammouda, Castro, Duran-Guerrero, Attia, & Azabou, 2023)
Kuqa apple	*L. plantarum*, *L. reuteri*, *L. rhamnosus* and *Pediococcus pentosaceus*	Obtained from China Microbial Culture Preservation Center (Beijing, China)	Mono-strain inoculation fermentation	*Pediococcus. pentosaceus* and *L. plantarum* are the most capable for fermenting Kuqa apple juice; *L. plantarum* increased SOD activity and DPPH radical scavenging activity	(Bai, Maimaitiyiming, & Wang, 2021)
Fresh Fuji apples	*L. plantarum* CICC21809	Obtained from the China Center of Industrial Culture Collection (CICC, Beijing, China).	Inoculation fermentation	Decreased pH; Increased total phenolic and tannic contents; Upregulated L-isoleucine, L-leucine, L-valine, 4-Guanidinobutyric acid, and Phenyllactate; Reduced diarrhea in mice through regulating gut microbiota, improving intestinal morphology, and increasing the expressions of AQP 1, 8, and TJ proteins	(Guo et al., 2022)
Lemon	*Issatchenkia terricola* WJL-G4	Isolated from the fresh fruits of red raspberry	Mono-strain inoculation fermentation	Reduced citric acid; Increased total phenolic and flavonoid contents; Increased antioxidant activities	(Liu, Wei, et al., 2021; Liu, Yuan, et al., 2021)
Grape	*Oenococcus oeni* UNQOe 73.2; *L. plantarum* UNQLp 11	Isolated from Patagonian Pinot Noir wines (vintages 2008 and 2012)	Mono-strain inoculation fermentation	Both strains increased the concentration of procyanidin and diminished the concentration of phenolic substances	(Brizuela et al., 2021)

(*continued on next page*)

Table 3 (*continued*)

Fruits	Strain	Strain source	Fermentation method	Activities	Reference
Cerasus humilis fruits	*L. plantarum*, *S. cerevisiae*	Not mentioned	Not mentioned	Increased flavonoid, phenolic, procyanidin, organic, and free amino acid; Decreased total sugar contents; Ameliorated hyperlipidemia and cholesterol over-accumulation; Relieved oxidative stress; Reversed fat deposition in high-fat diet rat liver; Increased the abundance of *Prevotella* and *norank_f_Muribaculaceae*	(Wang et al., 2023)
Fuji apples	*S. cerevisiae* CICC1750; *A. pasteurianus* CICC20056	Obtained from China microbial culture preservation Center	Inoculation fermentation	Reduced the release of inflammatory cytokines induced by mononuclear leukocyte infections; Increased monocyte phagocytosis	(Song et al., 2019)
Mulberry	*Levilactobacillus brevis* F064A	Isolated from Thai fermented sausage	Inoculation fermentation	Enhanced the growth of probiotics; Increased γ- aminobutyric acid content; Improved antioxidant activities; Exhibited lipid peroxidation inhibitory activity	(Kanklai et al., 2021)
Blueberries	*L. plantarum* J26	Isolated from traditional dairy products	Inoculation fermentation	Enhanced the scavenging abilities of DPPH, superoxide anion radical, and hydroxyl radical; Alleviated oxidative damage in the model of Caco-2 cells; Increased the inhibitory effect of α-glucosidase and α-amylase	(Zhang et al., 2021)
Five varieties of mango (*Baganpalli*, *Langra*, *Dashehari*, *Alphonso*, and *Totapuri*)	*S. cerevisiae* MTCC 178; Isolated yeast	*S. cerevisiae* MTCC 178 was from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh (India); Isolated yeast was from Department of Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi	Mixed-strain inoculation fermentation	Produced gallic acid, galloyl-A-type, proanthocyanidins, 2,2,6-trimethyl-6-vinyltetrahydropyran, β-pinene, and caffeoylquinic acid; Given potential antioxidant, anticancer, anti-inflammatory, and antibacterial properties.	(Patel, Tripathi, Adhikari, & Srivastava, 2021)
Grape	*Oenococcus oeni* MS9 and MS46	Isolated from wine collected from a cellar from Cafayate, Salta, Argentina	Mono-strain inoculation fermentation	Produced lactic acid to reduce pH; Increased total phenolic compounds with strain MS9 but not MS46; Increased antioxidant activity	(del Valle et al., 2022)
Hovenia dulcis	*S. cerevisiae*; *A. aceti*	Not mentioned	Inoculation fermentation	Increased DPPH and ABTS free radicals scavenging; Reduced power, hydrogen peroxide scavenging, and β-carotene bleaching activities; Reduced serum alcohol and acetaldehyde levels in SD rats administrated with 40% alcohol	(Park, Cho, Kim, Min, & Seo, 2023)
Fruit beverage prepared by kiwis, guavas, papayas, pineapples, and grapes	*S. cerevisiae* BCRC 21447; *L. acidophilus* BCRC 10695; *Pediococcus dextrinicus* BCRC 12842; *L. plantarum* BCRC 10069; *A. pasteurianus* BCRC 14145	Purchased from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan)	Mixed-strain inoculation fermentation	Reduced calorie intake; Enhanced phagocytosis and T cell proliferation; Enhanced proinflammatory cytokines production; Decreased the production of pro-inflammatory cytokines in OVA-immunized mice	(Sy, Hsu, Limaye, & Liu, 2020)
Gilaburu (*Viburnum opulus*)	*L. plantarum*; *L. delbrueckii*; *L. caseii*	Obtained from microbiology laboratory of Adana Alparslan Turkes Science and Technology University in Turkey	Natural or inoculation fermentation	Increased phenolics and volatiles via inoculation fermentation	(Sevindik et al., 2022)

L. fermentum, *Lactobacillus fermentum*; *L. acidipiscis*, *Lactobacillus acidipiscis*; *L. salivarius*, *Lactobacillus salivarius*; *L. reuteri*, *Lactobacillus reuteri*; *A. niger*, *Acetobacter niger*; *L. delbrueckii*; *Lactobacillus delbrueckii*.

difficult to control, especially during long-term storage and transportation; 3) The quality of fermented fruit varies greatly; 4) The quality of products fermented by mono-strain fermentation is poor; 5) The synergistic effect of multiple strains and the control of fermentation process are challenges.

5. Prospects

Microbial fermentation has emerged as a prominent focus within the interdisciplinary fields of medicine and nutrition. With a growing emphasis on health, fermented fruit products have become an important part of the functional food market. Microbial fermentation can not only reduce the spoilage of fruits and improve their nutritional value, but also realize the high-value utilization of fruit processing by-products such as peel, pomace, and kernel. By summarizing the common strains applicable to fruit fermentation, common methods for fruit fermentation, and the effects of fermentation on fruit quality such as shelf-life, flavor, and functional activity, this review aims to provide a diverse theoretical basis for food innovation. Although fruit fermentation can organically combine the nutrients of fruit, probiotics, and their active metabolites-prebiotics (polyphenols, polysaccharides, and dietary fibers), the development of fruit fermentation products is still in the primary stage. There are the following points need to be studied: 1) to screen excellent microbial strains giving full play to the fruit and microbial health effects; 2) to improve fermentation technology; 3) to establish relevant industry standards proving the safety of fermented fruit products; 4) to develop fermented fruits with rich and diverse flavors meeting the different needs of people; 5) to strengthen the functionality of fermented fruit

products meeting the needs of special populations.

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CRediT authorship contribution statement

Xinyu Yuan: Writing – original draft, Investigation, Data curation. **Tao Wang:** Writing – review & editing, Methodology. **Liping Sun:** Writing – review & editing, Investigation. **Zhu Qiao:** Writing – review & editing, Investigation. **Hongyu Pan:** Investigation. **Yujie Zhong:** Writing – review & editing, Project administration, Investigation. **Yongliang Zhuang:** Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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