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## Screening of antibacterial activity of lactic acid bacteria isolated from fermented vegetables against foodborne pathogenic bacteria and investigation of their potential production of bacteriocins

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**Abstract** A total of 9 lactic acid bacteria (LAB) strains were isolated and purified from 4 different fermented vegetables (3 strains obtained from cabbage, 3 strains from artichoke, 2 from cauliflower and 1 from carrot). All strains were Gram positive including 2 cocci and 7 rods cell morphology with different cellular arrangement. Our results of physiological and biochemical tests revealed that all strains were catalase negative and only one strain out of 9 of these isolates showed positive acetoin production. All strains were grown at 15 °C, 45 °C and pH 9.6. The capacity of LAB isolates to grow at different salt concentrations was evaluated. All isolates, with the exception of strain 9, exhibited growth at 2% and 4% NaCl. Growth at 6.5% NaCl was observed in isolates 1, 2, and 7, whereas isolates 3, 4, 5, 6, 8, and 9 did not grow at this concentration. In addition, the cell-free supernatant produced by the nine LAB isolates exhibited antimicrobial activity against both Gram-positive and Gram-negative foodborne bacteria with inhibition zones ranged between (12.50- 18.33 mm) against *Staphylococcus aureus* ATCC 25923, (11.00-15.00 mm) against *Bacillus cereus* ATCC 25923, (12.00-15.00 mm) against *Pseudomonas aeruginosa* ATCC 27853 and (10.66-15.00 mm) toward *Escherichia coli* ATCC 25923. Moreover, the LAB isolates did not produce bacteriocin-like compounds. Therefore, the antibacterial activity is mainly due to the production of organic acids and H<sub>2</sub>O<sub>2</sub>. The finding underlined the important role of LAB strains in improving food quality and increasing safety and their interesting useful in the food industry instead of chemical preservatives.

**Keyword:** Antibacterial activity, Lactic acid bacteria, Foodborne pathogenic bacteria, Bacteriocins

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## Introduction

Lactic acid bacteria (LAB) are a heterogeneous group of Gram-positive bacteria, rods or cocci, nonsporing, nonrespiring, and produce lactic acid as the primary product of carbohydrates fermentation. This large group includes *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Bifidobacterium* and *Leuconostoc* (Masood *et al.*, 2011; Bintsis, 2018). These bacteria play a crucial role in agricultural, food and medical applications, they are employed in industrial fermentations to enhance texture, flavour and nutritional value of the fermented foods. Indeed, the fermentation process enhances the shelf life and microbiological safety of food, improves digestibility, and, in the case of cassava, reduces substrate toxicity. Their frequent presence in foods along with their long history of use makes them naturally accepted as GRAS (Generally Recognised as Safe) for consumption by humans (Caplice and Fitzgerald, 1999; Mokoena *et al.*, 2011; Bintsis, 2018). They are commonly found in the intestinal tracts of diverse animals as part of the normal flora. *Lactobacillus* is the largest genus within this order comprising nearly 80 species that are used in various products including pickles, beer, juices, sauerkraut, wine, yogurt, cheese and sausage (Masood *et al.*, 2011). Today, fermented vegetables and fruits play a significant role in feeding populations worldwide. They produce beneficial and nutritious foods with a wide range of flavors, aromas and textures. Moreover, the fermentation process for vegetables and fruits produce foods that are storable for one year or more without refrigeration, contrary to fresh ones which are susceptible to various microorganisms, such as aerobic spoilage microflora (*Pseudomonas*, *Erwinia*, and *Enterobacter* species), alongside yeasts and molds (Breidt *et al.*, 2013; Swain *et al.*, 2014), since LAB used in vegetables can produce an important range of metabolites with antimicrobial action, including lactic acid, acetic acid, hydrogen peroxide and many low molecular weight substances (fatty acids, reutericyclin, reuterin and diacetyl), antifungal compounds (bacteriocins, propionate, phenyl lactate, hydroxyphenyl lactate), therefore, they are very useful in the food sector against foodborne pathogens and microorganisms that cause food spoilage including viruses (Castellano *et al.*, 2017). Bacteriocins are polypeptides produced by bacteria through ribosomal synthesis. They vary significantly in terms of molecular weight, biochemical properties, and range of susceptible hosts and mechanisms of action. They can have a bacteriocidal or bacteriostatic effect against species related with the producing strain. There are two main categories of bacteriocins. Class I bacteriocins including lactococcin, primarily act by inhibiting the synthesis of peptidoglycan. Class II bacteriocins, such as nisin, act by the creation of pores by destabilizing the cytoplasmic membrane. Thus, they are very useful in food fermentations, as they can prevent spoilage and inhibit food pathogens. Bacteriocins are powerful and

promising antimicrobials that are highly attractive for the food production because of their thermal stability, proteinaceous nature and safety for human consumption. Thus, LAB strains producing bacteriocins are very promising for food safety applications (Soomro *et al.*, 2002; Bintsis, 2018; Ibrahim *et al.*, 2021; Zapa'snik *et al.*, 2022). In this context, the aim was evaluated the antibacterial activity of some LAB strains isolated from fermented vegetables and investigation of their potential production of bacteriocins.

## **Material and methods**

### ***Lactofermentation of vegetables***

The lactofermentation of four types of vegetables (cabbage, cauliflower, artichoke and carrot) was performed using traditionally method. The process involved immersing vegetables, naturally rich in lactic acid bacteria, in a brine solution of water and salt for 7-14 days within an airtight container, such as a glass jar, ceramic crock, or food-grade plastic container, to minimize oxygen exposure.

### ***Isolation of LAB***

Serial decimal dilution ranging from  $10^{-1}$  to  $10^{-6}$  was prepared by diluting 1 mL of vegetable juice in 9 mL of physiological saline (0.9% NaCl). From each dilution, 0.1 mL was spread directly onto the surface of MRS agar plates containing 1% CaCO<sub>3</sub>. After transferring bacterial culture, the agar plates were placed in an anaerobic environment using an anaerobic candle jar and then incubated for 48 hours at 37°C. Lactic acid bacteria colonies were distinguished by a clear zone surrounding each colony. Randomly selected colonies from MRS plates were purified through successive streaking on MRS agar. Gram-positive and catalase-negative bacterial isolates were further purified and stored in 10% (w/v) skim milk at -4°C. (Chen *et al.*, 2010; Yu *et al.*, 2011).

### ***Physiological examination***

Gram-positive and catalase-negative isolates were further identified using the following physiological tests such as growth at different ambient temperatures (15 and 45 °C), the growth at different NaCl concentrations 2.5%, 4.0% and 6.5%NaCl (w/v) and the growth at pH 9.6 (Goa *et al.*, 2022).

### ***Acetoin production (VP)***

The ability of strains to produce aromatic compounds (acetoin) during the fermentation process was demonstrated on medium Clark and Lubs. Each tube containing 5ml of sterile medium was transferred to each lactic acid bacteria isolate. After incubation for 24 hours at 37°C, the reagents of Vogues-Proskauer VPI (NaOH 16% alcohol) and VPII (alpha-naphthol 6% of alcohol) were added to the cultures (V/V) and the mixture is maintained for 10min before reading the reaction. The presence of a pink ring on the surface of the culture shows the positive response (Moulay *et al.*, 2013).

### ***Testing bacteria***

To detect the antibacterial activity of LAB isolates, four strains of foodborne pathogens bacteria obtained from the National Center for Biotechnology Research (Constantine, Algeria) were used: *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC11778, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922.

### ***Antibacterial effect of LAB***

The antibacterial activity of LAB was assessed to determine combined impact of antibacterial agents against the four strains of foodborne pathogens. Antibacterial activity of lactic acid bacteria was tested using agar well diffusion method. LAB isolates were transferred to tubes containing 5 ml of MRS broth and incubated at 37°C for 24 h. The MRS broth tubes were then centrifuged (10,000 g, 10 min) to generate cell free supernatants (CFS). The CFS was then filtered through a sterile 0.22 µm syringe filter. The indicator bacterial strains were collected on prepared nutrient agar slants, sub-cultured on nutrient agar plates, and incubated for 24 hours at 37°C. Prior to use, the bacterial cultures were standardized to a 0.5 McFarland turbidity standard in 0.85% saline solution. Each Mueller-Hinton agar plate was thoroughly swabbed with indicator bacterial strains using sterile cotton swabs. Wells of 6 mm in diameter were then punched into the agar using sterile yellow Pasteur pipette tips, and 100 µl of the cell-free supernatant (CFS) from LAB isolates was dispensed into each well. The transferred media were incubated at 37° C for 24 hours. After incubation, the plates were observed for a zone of inhibition (ZOI) around the well. The ZOI diameter, expressed in mm was assessed. LAB strains exhibiting ZOI below 11 mm, ranging from 11-16 mm, 17-22 mm, and exceeding 23 mm were designated as negative (-), mild (+), strong (++), and very strong (+++) inhibitors, respectively (Zare Mirzaei *et al.*, 2018; Goa *et*

*al.*, 2022). The experiments were carried out in triplicates and the mean values of three readings were recorded.

### ***Production of bacteriocin-like compounds***

In order to screen bacteriocin producing bacteria, the pH of the CFS was measured and adjusted to 6.5 with NaOH (1M) to exclude the inhibitory effect of organic acid. The CFS was then treated with catalase (1 mg/ml, Sigma-Aldrich, Germany) and incubated at 25°C for 1 h to eliminate possible inhibitory action of H<sub>2</sub>O<sub>2</sub> and filtered. The antibacterial activity of the CFS of each isolate was tested using the agar well diffusion assay (100 microliters of each CFS was poured into each well) (Zare Mirzaei *et al.*, 2018; Goa *et al.*, 2022). The experiments were conducted in triplicate, and the average values of three measurements were recorded.

## **Results**

### ***Isolation and characterization of LAB***

A total of 9 LAB strains were isolated and purified from 4 different fermented vegetables (3 strains obtained from cabbage, 3 strains from artichoke, 2 from cauliflower and 1 from carrot). All strains were Gram positive including 2 cocci and 7 rods cell morphology with different cellular arrangement (Table 1).

**Table 1.** Microscopic characteristics of lactic acid bacteria isolated from fermented vegetables

<b>Strain No.</b>	<b>Source</b>	<b>Gram</b>	<b>Cell morphology</b>	<b>Cellular arrangement</b>
1	Cabbage	+	Rod	Single, paired and short chained Bacilli
2	Cabbage	+	Rod	Single, paired and long chained Bacilli
3	Artichoke	+	Rod	Single, diploid, tetraploid and short chained Bacilli
4	Cabbage	+	Rod	Single and long chained Bacilli
5	Cauliflower	+	Rod	diploid, tetraploid
6	Cauliflower	+	Cocci	diploid, tetraploid and short chained cocci
7	Artichoke	+	Rod	Single, diploid and tetraploid
8	Artichoke	+	Cocci	diploid, tetraploid and short chained cocci
9	carrot	+	Rod	Diploid, tetraploid and short chained Bacilli.

Based on the physiological and biochemical test results, all strains were found to be catalase negative and only one strain out of 9 of these

isolates showed positive acetoin production (Table 2). All strains were grown at 15 °C, 45 °C and pH 9.6. The growth potential at various salt concentrations indicated that all LAB isolates exhibited growth at NaCl concentrations of 2% and 4%, except for strain 9, which was not grown at 4% NaCl. LAB isolates 1, 2 and 7 were grown at 6.5% NaCl concentration, while the growth of isolates 3, 4, 5, 6, 8 and 9 were not observed at 6.5 % NaCl.

**Table 2.** Biochemical and physiological characteristics of lactic acid bacteria isolated from fermented vegetables

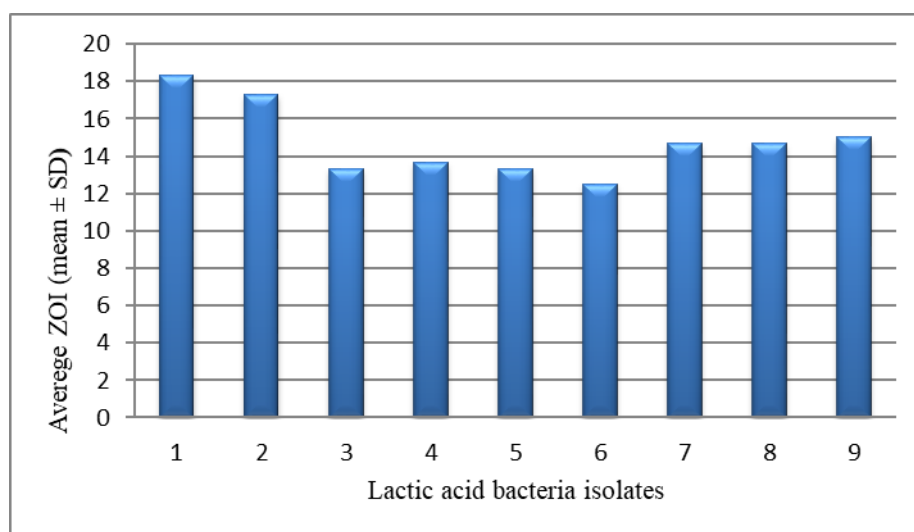
Strain.No	Catalase	VP	Growth at different NaCl concentrations			Growth at different temperature		at growth at pH 9,6
			2.5%	4%	6,5 %	45° C	15° C	
1	-	-	+	+	+	+	+	+
2	-	+	+	+	+	+	+	+
3	-	-	+	+	-	+	+	+
4	-	-	+	+	-	+	+	+
5	-	-	+	+	-	+	+	+
6	-	-	+	+	-	+	+	+
7	-	-	+	-	+	+	+	+
8	-	-	+	+	-	+	+	+
9	-	-	+	+	-	+	+	+

### ***Antibacterial activity of LAB isolates against foodborne pathogens bacteria***

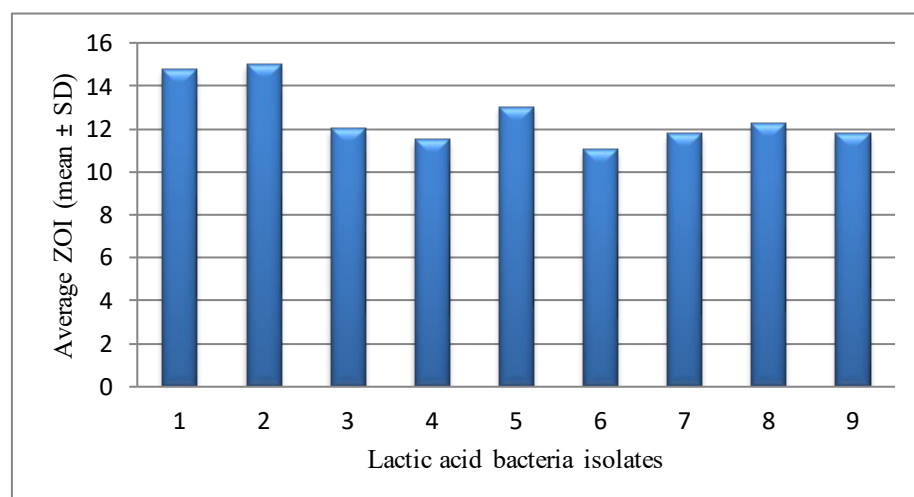
Nine LAB isolates from fermented vegetables were tested for their antibacterial activity against foodborne pathogens (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 and *Bacillus cereus* ATCC11778) using agar well diffusion method.

The diameter of inhibition zones showed that all isolates significantly inhibited the growth of all tested bacteria. The CFS from the nine LAB isolates exhibited an antibacterial activity against *S. aureus* ATCC 25923 (Figure 1). The isolates 1 and 2 showed a strong antibacterial activity with ZOI means  $18.33 \pm 0.57$  and  $17.33 \pm 1.15$  mm respectively, while the other isolates (3, 4, 5, 6, 7 and 8) showed mild inhibitory effect with ZOI means corresponding to  $13.33 \pm 0.57$ ,  $13.66 \pm 0.57$ ,  $13.33 \pm 0.57$ ,  $12.5 \pm 0.57$ ,  $14.66 \pm 1.52$ ,  $14.66 \pm 0.57$ ,  $15 \pm 0$  mm respectively. All LAB showed a mild antibacterial activity against *B. cereus* ATCC11778 with ZOI means of  $14.75 \pm 0.5$ ,  $15 \pm 1$ ,  $12 \pm 0.81$ ,  $11.5 \pm 1.29$ ,  $13 \pm 1.82$ ,  $11 \pm 1.15$ ,

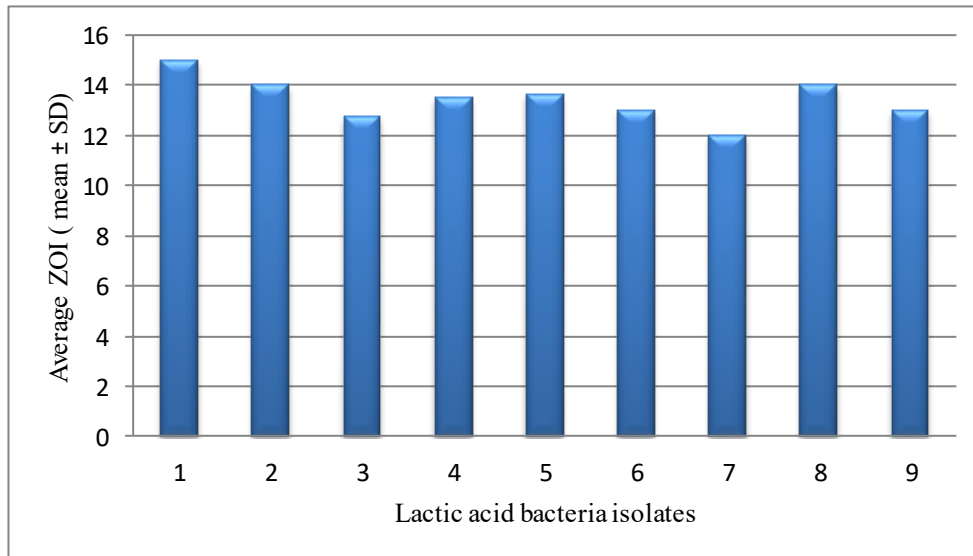
11.75±0.5, 12.25±0.5 and 11.75±1.70 mm respectively (Figure 2). Lactic acid bacteria isolate 1 was effectively inhibited *P. aeruginosa* ATCC 27853 with ZOI mean of 15 ±0mm, followed by LAB isolates 2, 8, 5, 4, 6, 9, 3 and 7 with ZOI means of 14±0.81, 14±0.81, 13.66±0.57, 13.5±0.57, 13±0.13, 13±0.81 and 12±0.81 mm respectively (Figure 3). For Lactic acid bacteria isolate 1 was also effectively inhibited *E. coli* ATCC 25922 with inhibition zone of 15 ±0 mm. The other LAB isolates 2, 3, 4, 5, 6, 7, 8 and 9 exhibited a mild antibacterial activity with ZOI means of 14.75 ±0.95, 11.5 ±0.57, 11 ±0, 10.66±0.57, 10.66±0.57, 12.66±0.57, 13±1 and 13.25±0.95 respectively (Figure 4).



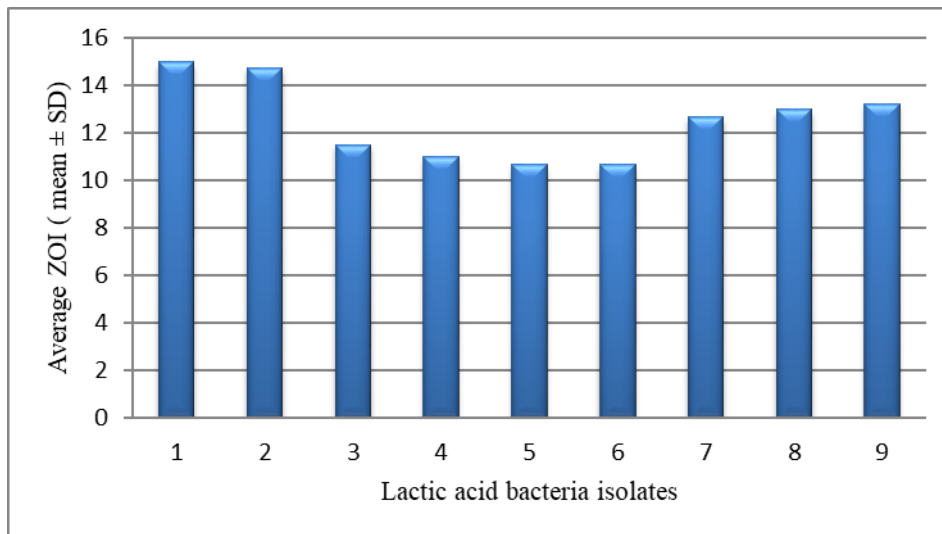
**Figure 1.** Average inhibition zones of LAB isolates against *Staphylococcus aureus* ATCC 25923



**Figure 2.** Average inhibition zones of LAB isolates against *Bacillus cereus* ATCC 25923



**Figure 3.** Average inhibition zones of LAB isolates against *Pseudomonas aeruginosa* ATCC 27853



**Figure 4.** Average inhibition zones of LAB isolates against *Escherichia coli* ATCC 25923

***Production of bacteriocin-like compounds***

The antibacterial activity of all LAB isolates disappeared once we balanced the pH of the culture filtrate supernatant (CFS) to 6.5, which eliminated the inhibitory impact of organic acids. Furthermore, even after treating the CFS with catalase to remove any potential inhibitory action of



H<sub>2</sub>O<sub>2</sub>, the antimicrobial activity remained absent. This suggests that the LAB isolates did not produce compounds resembling bacteriocins.

## Discussion

Foodborne pathogens cause a variety of diseases and have major impacts on human health and economy. The occurrence of foodborne illness arises from the ingestion of pathogens, which subsequently colonize the human host and may proliferate, or from the contamination of food by toxigenic pathogens, resulting in the production of toxins that are subsequently ingested by humans (Bintsis, 2017). Lactic acid fermentation is anticipated to become a significant method to play a crucial role for preserving and ensuring the microbial safety of fruits, fresh vegetables, and other food items, especially in developing nations. This technique helps to maintain the stability of the final fermented products by promoting microbial stability. Numerous studies have investigated the use of by-products generated by lactic acid bacteria (LAB) to inhibit the growth of food-borne pathogens, particularly in light of the ongoing emergence of multi-drug resistant strains. Moreover, LAB strains also can reduce fungal mycotoxins, either by absorbing them, or by producing anti-mycotoxinogenic metabolites (Swain *et al.*, 2014; Bintsis, 2018; Irorita Fugaban *et al.*, 2022). Several studies reported that certain LAB strains exhibited the capacity to restrain the proliferation of specific pathogenic microorganisms in food products (Schoˆbitz *et al.*, 1999; Callewaert *et al.*, 2000; Awaisheh and Ibrahim, 2009; Girma and Aemiro, 2021).

In this study, 9 lactic acid bacteria strains isolated from four types of fermented vegetables (cabbage, cauliflower, artichoke and carrot) were screened for their antibacterial activities against four foodborne pathogenic organisms (*B. cereus* ATCC11778, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853). All LAB strains CFS showed antibacterial effects against the tested bacteria. Breidt *et al.* (2013) reported that, compared with other mesophilic microorganisms, LAB was initially presented in fresh vegetables in lower amounts, 10<sup>2</sup>-10<sup>3</sup> CFU/g. During fermentation, the diffusion of organic acids into the brine and the resulting low pH affected the growth of microorganisms on the surface of the plant material. LAB grew rapidly due to the diffusion of sugars from the vegetables into the brine. LAB are more acid-tolerant than spoilage microbiota, they dominated the fermentation of pickles. Growth of spoilage microbiota unhindered by LAB metabolic in the absence of brine. Moreover, LAB produce a variety of antimicrobial compounds, exerting antagonistic effects on the growth of specific spoilage and pathogenic bacteria in food. These substances fall under the classification of low-molecular-weight (LMM) compounds, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and diacetyl (2,3-butanedione), carbon dioxide (CO<sub>2</sub>), and high-molecular-

mass (HMM) compounds like bacteriocins (Jay, 1982; Klaenhammer, 1988; Piard and Desmazeaud, 1991; Ammor *et al.*, 2006). The antimicrobial effect of organic acids produced at the end of fermentation against contaminant microorganisms lies in the reduction of pH, which contributed to the dissociation of the acid. The decrease in external pH results in the acidification of the cell cytoplasm. Simultaneously, the undissociated acid, being lipophilic, may diffuse passively through the membrane. This diffusion can disrupt the electrochemical proton gradient or alter cell membrane permeability, thereby interfering with substrate transport systems. (Snijders *et al.*, 1985 ; Kashket, 1987; Ammor *et al.*, 2006; Irorita Fugaban *et al.*, 2022). Wang *et al.* (2015) indicated that, after exposure to 0.5% lactic acid for 2 h, *S. enteritidis*, *E. coli* and *L. monocytogenes* cells could be completely inactivated as result of a great leakage of their proteins cells. CO<sub>2</sub> demonstrates effective inhibition against the proliferation of numerous food spoilage microorganisms, particularly Gram-negative psychrotrophic bacteria. The inhibitory action of CO<sub>2</sub> is due to the formation of an anaerobic environment, which suppresses enzymatic decarboxylations, and the accumulation of CO<sub>2</sub> within the lipid bilayer of the membrane can cause disruptions in its permeability (Ammor *et al.*, 2006). The hydrogen peroxide production in the presence of oxygen molecules in lactic acid bacteria (LAB) is due to the activity of flavoprotein-containing oxidases, NADH oxidases, and superoxide dismutase. The antimicrobial effect of H<sub>2</sub>O<sub>2</sub> leads to the oxidation of sulfhydryl groups causing denaturing of a number of enzymes and from the peroxidation of membrane lipids as result of increasing membrane permeability (Kong and Davison, 1980). The ability of diacetyl to interact with the arginine-binding proteins of the target cells is the responsible of the antimicrobial activity, which may affect the protein utilization of the target organisms. This activity is more effective against Gram-negative bacteria (Irorita Fugaban *et al.*, 2022). The antibacterial effect of bacteriocins is frequently due to the cell death as result of their disruption of bacterial membrane integrity. This effect may arise from direct interactions with the lipid II component of the bacterial membrane, the mannose phosphotransferase system (Man-PTS), or independent of specific receptor involvement (Simons *et al.*, 2020). The current study showed that the cell-free supernatant produced by the nine LAB strains isolated from fermented cabbage, cauliflower, artichoke and carrot, exhibited antibacterial activity toward both Gram-positive and Gram-negative foodborne bacteria. This antibacterial activity disappeared when the pH of CFS was adjusted at 6.5 and treated with catalase. Therefore, the antibacterial activity is mainly due to the production of organic acids, H<sub>2</sub>O<sub>2</sub> and diacetyl. The finding is underlined the important role of LAB in improving food quality and increasing safety as well as their interesting useful in the food industry instead of chemical preservatives.

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