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Characterization and Optimization of Bacteriocin from *Lactobacillus plantarum* Isolated from Fermented Beef (Shermout)

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Abstract

Many lactic acid bacteria (LAB) were isolated from "Shermout", a popular Sudanese fermented beef product intended for long storage. An isolate that demonstrated significant antibacterial activity was identified as *Lactobacillus plantarum* PM4 based on phenotypic, physiological and biochemical characteristics and carbohydrate utilization patterns. The inhibitory activity of the partially purified bacteriocin was completely arrested by the proteolytic enzymes proteinase-k and pepsin but not by α-amylase, asserting its proteinaceous nature. The activity was not due to H_2O_2 as similar inhibition was obtained by cell-free supernatant (CFS) produced under anaerobic conditions. The bacteriocin showed a molecular weight in the range of 3 - 5 kDa and had a bactericidal mode of action. No significant reduction in activity was observed on heating to 60˚C for 60 min, but activity was lost on heating to 100˚C or autoclaving. Highest inhibitory activity was at pH 5.5 and there was appreciable reduction in activity at pH 3, 7 or 9. There was no drop in activity at −80˚C or −20˚C up to four weeks of storage. However, at 4˚C and 35˚C, a gradual decline in activity was observed. *Lb. plantarum* PM4 exhibited bactericidal activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli* ATCC25922, *Klebsiella pneumoniae* and *Proteus vulgaris*. Bacteriocin production generally coincided with the phase of maximum growth and the best combination for maximum production of inhibitory activity was at pH 5.5 for 48 h whether incubated at 25˚C, 30˚C or 37˚C. *Lb. plantarum* PM4 showed promise as a starter culture in the fermentation of preserved meat products.

Keywords

Lactobacillus plantarum, Bacteriocin, Fermented Beef

1. Introduction

Lactic acid bacteria (LAB) is a group of Gram positive facultative anaerobic bacteria that are able to produce antagonistic molecules in their growth medium that can be used as antimicrobials and preservatives. These antagonistic properties of LAB are allied to their safe history of use in traditional fermented food products that make them very attractive as biopreservatives that can replace or allow reduction of chemical additives [1]. LAB is used in food biopreservation because they are safe for human consumption enjoying the status of GRAS (Generally Recognized as Safe) and are the prevalent indigenous microflora in many foods. Accordingly, a wide variety of LAB strains are routinely employed as starter cultures in the manufacture of meat, dairy, vegetable and bakery products [2] [3]. One of the most important contributions of these bacteria—whether indigenous or added as starters—is the extension of shelf life of the fermented products through inhibition of the growth of spoilage and pathogenic bacteria in these foods due to competition for nutrients and the presence of the antagonistic molecules such as lactic acid, hydrogen peroxide, diacetyl and bacteriocins [4]. Moreover, health benefits acclaimed to be offered by LAB include production of vitamins, immunomodulation, reduction in the risk of diarrhea, and a decrease in serum cholesterol [5] [6] [7]. Among the antagonistic molecules produced by LAB are bacteriocins [8] which are antimicrobial peptides or proteins produced by strains of diverse bacterial species. The antimicrobial activity of this group of natural substances against foodborne pathogens, as well as spoilage bacteria, has raised considerable interest for their application in food preservation [2] [9] [10] [11].

Within LAB, the lactobacilli are an important group recognized for their fermentative ability as well as health and nutritional benefits [12]. In this group, *Lactobacillus plantarum* is one of the most widely distributed in nature, and is one of the most versatile species, used both as starter and probiotic [13] [14]. *Lb. plantarum* has been isolated from various habitats, and bacteriocins have been described for strains from fermented meat products [15] [16] [17]. It is one of the most important LAB strains used for the production of fermented meat products [18]. Over the past few decades, there has been an increasing research interest in the development of nitrite-free meat curing systems. The principle concern with the use of nitrite for curing of meat is the eventual formation of carcinogenic N-nitrosamines [19]. Consumers are increasingly demanding food that is free from pathogens, with minimal processing and fewer chemical preservatives and additives. Thus biopreservation has gained increasing attention as means of naturally controlling the shelf life and safety of meat products. In recent years bacteriocins of lactic acid bacteria have attracted the attention of

many investigators because of their use as a natural food preservative with probiotic capability within the human body after ingestion of food [14] [20].

Shermout is sun-dried lean beef strips widely used for prolonged storage. It has unique sensory characteristics and is very popular in Sudan and neighboring countries. It is very similar to "kaddid" [17] and jerky [21] [22] except that no or little salt is added and the product undergoes mild fermentation by indigenous microbial flora, mainly LAB. The process is artisanal in nature, with no bacterial starters added and is usually subject to microbial deterioration. Various gram positive and gram negative bacteria like *Salmonella typhi*, *Bacillus subtilis* and staphylococci are the main causative organisms [23]. The objectives of this study were the isolation and identification of *Lb. plantarum* from local Sudanese fermented beef (shermout), characterization of the bacteriocin it produces and determination of its antibacterial activity, study of the bacteriocin kinetics and determination of the optimum growth conditions for bacteriocin production.

2. Materials and Methods

2.1. Isolation of the Bacteriocinogenic Bacterium

Ten g of traditional Sudanese fermented beef (shermout) samples were aseptically added to 90 mL sterile peptone water (10 g peptone/L distilled H_2O), carefully shaken and were left to homogenize for I h. Serial decimal dilutions were prepared from the sample homogenate, and were streaked onto duplicate plates of MRS medium [24] to which 0.1% (w/v) nystatin had been added to inhibit fungal growth [25]. The streaked plates were incubated anaerobically at 30˚C for 2 - 3 days in an anaerobic jar system (GasPak; BBL Microbiology Systems, Cockeysville, Maryland, USA) with a gas-generating kit (BR0038B, Oxoid, Hampshire, UK). The pure colonies obtained were examined for Gram reaction, catalase activity, and spore formation. A Gram-positive, catalase-negative, non-spore-forming rod was selected and identified as *Lactobacillus plantarum* by use of the fermentation pattern from KB009 HiCarbohydrate identification kit (Hi-Media Laboratories, Mumbai, India) in conjunction with other tests which included growth at 10 and 45 C, tolerance of 6.5% NaCl, pH (4.4 and 9.6) and gas production from glucose.

2.2. Production, Partial Purification and Characterization of Supernatant from *Lb. plantarum* PM4

For partial purification of the cell-free supernatant (CFS), a modification of the method of ten Brink *et al.* (1994) [26] was adopted. Sensitivity of the CFS to various enzymes (proteinase K, pepsin, α-amylase) was conducted [27]. Production of the inhibitory factor during anaerobic growth was investigated following the technique detailed in [28]. The effects of heating (40˚C, 60˚C, 100˚C for 10, 30 and 60 minutes in addition to autoclaving at 121˚C and 15 psi) and different pH values (3.0, 5.0, 7.0 and 9.0) were tested according to [29]. The stability of the antagonistic activity of the CFS during storage (−80˚C, −20˚C, 4˚C and 35˚C for 4 weeks) was also tested. The retained activity in all tests was determined using

the agar well diffusion test utilizing *Staphylococcus aureus* ATCC43306 as the indicator organism.

For molecular weight determination, the active moiety in the CFS was first precipitated by addition of 40% (w/v) ammonium sulfate, centrifuged (6000 rpm for 15 minutes), and the pellet and pellicle were concentrated and used for protein separation by tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) using 15% acrylamide. The gel electrode assembly was placed in a Mini PROTEAN II electrophoresis chamber (BIORAD), and the protein was electrophoresed at 100 volts for about 2 h. A protein molecular weight marker (MoBiTec GmbH) with a molecular weight range of 14.0 to 116.0 KDa was included.

Staining of the gels was done by covering with Coomassie blue stain overnight and the preparation was destained with a buffer composed of 10.0 ml glacial acetic acid, 50.0 ml methanol and 100 ml deionized water.

2.3. Mode of Action

This test was conducted to find out whether the antagonistic effect of the CFS was bactericidal or bacteriostatic in nature. The procedure followed was similar to that described in [30] and [31].

2.4. Spectrum of Activity

The inhibitory activity was tested against eight indicator bacteria, namely: *S. aureus* ATCC 25923, *Enterococcus faecalis* ATCC 10541, *Escherichia coli* ATCC 25922, *E. coli* (local isolate), *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 10031, *Proteus vulgaris* ATCC 6380 and *Salmonella typhi* ATCC 1319106. The antagonistic activity was determined by the well diffusion method in Nutrient Agar plates. Diameters of the inhibition zones were measured after 24 h of incubation at 35˚C.

2.5. Kinetics of Growth and Bacteriocin Production

The kinetics of growth and production of the antagonistic activity was investigated using the procedure described by [32]. MRS broth (250 mL) was inoculated with 1% of an overnight culture of the strain and incubated at 30˚C without agitation under uncontrolled pH conditions. Samples were removed at hourly intervals up to 13 h, and then at 24, 25 and 26 h from start of the investigation. Measurement of biomass by absorbance at 600 nm, pH measurement and determination of the antibacterial activity were carried out by assaying the effect of serial two-fold dilutions of the CFS on *Staphylococcus aureus* ATCC 43306 by the well-diffusion method. The antimicrobial titer was expressed in arbitrary units (AU/mL). One arbitrary unit was defined as the reciprocal of the highest dilution showing a clear inhibition zone around the well [33].

2.6. Optimization of Growth and Bacteriocin Production

For determination of the effects of incubation temperature, pH of the growth

medium and incubation period on bacteriocin production, three levels of each of these three factors were chosen and tested. The temperature levels were 25˚C, 30˚C and 37˚C, the pH values were 5.0, 5.5 and 6.0 while the incubation times were 24, 48 and 72 h. The test isolate was grown in MRS broth. Growth was measured as optical densities (O.D.) at the wavelength of 600 nm, and inhibitory activity was measured in arbitrary units (AU).

3. Results and Discussion

3.1. Isolation and Screening of the Bacteriocinogenic Bacterium

A total of 39 isolates of antagonistic LAB were obtained from Sudanese fermented beef. All isolates were Gram-positive, catalase-negative, non-spore forming rods or cocci capable of growth under anaerobic conditions, conforming to the characteristics of lactic acid bacteria [34]. An isolate that gave positive result in the preliminary screening (spot on lawn method) against indicator bacteria (*Staphylococcus aureus* ATCC 43306, *Bacillus subtilis* NCTC 8236) with good inhibition zone diameter (well diffusion method) was selected and identified as *Lactobacillus plantarum* PM4. The inhibition zone by the selected isolate was produced as early as 24 h. The isolate was rod shaped, did not grow at 10˚C but grew at 45˚C. It grew at pH 4.4 but not at pH 9.6. It did not grown in the presence of 6.5% of NaCl, and could not hydrolyse arginine. It therefore belonged to the genus *Lactobacillus* (homofermentative lactobacilli) [34] [35] [36] [37]. **Table 1** shows the pattern of utilization of 35 sugars by the isolate based on which it was identified as *Lactobacillus plantarum* PM4 [38] [39].

3.2. Characterization of the Cell-Free Supernatant (CFS)

The partially purified CFS from *Lb. plantarum* PM4 was subjected to various treatments. **Table 2** shows effects of the enzymes proteinase-k, pepsin and *α*-amylase on the CFS. No inhibition was produced by the CFS in presence of the protein-digesting enzymes (proteinase-k and pepsin) indicating complete destruction of the inhibitory substance in the CFS, and asserting its proteinaceus nature; while no reduction in the inhibitory activity was observed in the presence of the carbohydrate-degrading *α*-amylase. No inhibitory activity was shown in the uninoculated medium containing no enzyme (negative control). This suggested that the antibacterial activity was associated with bacteriocin [40] [41]. Treatment with a-amylase did not affect the inhibitory activity suggesting that the CFS, similar to most other bacteriocins, was not glycosylated [38] [39] [42] [43].

As production of hydrogen peroxide under aerobic conditions is one of the potent defense weapons of LAB, elimination of this factor was achieved through growing the isolate under anaerobic conditions. There was no difference in inhibitory activity against indicator bacterium whether *Lb. plantarum* PM4 was grown under aerobic or anaerobic conditions (result not shown), indicating that the inhibitory activity was not due the production of H_2O_2. **Figure 1** shows that the widest inhibition zone of the CFS from *Lb. plantarum* PM4 on *Staph. aureus*

ATCC 43306 (20 mm) occurred at pH 5.0, declining gradually with either increase or decrease in pH value.

No reduction was observed in the inhibitory activity of the CFS on heating to 40˚C for 10 or 30 min, but a slight reduction was observed when the heating was continued for 60 minutes. However, a 7.7% reduction in activity was observed on heating at 60˚C, whether for 10, 30 or 60 minutes, while heating to 100˚C or 121˚C resulted in complete loss of the activity regardless of the length of the heating period (**Figure 2**). Loss of activity after heat treatment at 121˚C for 15 min has been reported [44].

Table 1. Carbohydrate utilization pattern by *Lb. plantarum* M4.

Carbohydrates	Utilization	Carbohydrates	Utilization
Lactose	+	Glucosamine	+
Xylose	–	Dulcitol	+
Maltose	+	Inositol	+
Fructose	+	Sorbitol	+
Dextrose	+	Mannitol	+
Galactose	+	Adonitol	–
Raffinose	+	α-Methyl-D-glucoside	+
Trehalose	+	Ribose	+
Melibiose	+	Rhamnose	–
Sucrose	+	Cellobiose	+
L-Arabinose	+	Melezitose	+
Malonate utilization	+	α-Methyl-D-mannoside	+
Mannose	+	Xylitol	+
Inulin	+	ONPG	–
Sodium Gluconate	+	Esculin hydrolysis	–
Glycerol	+	D-Arabinose	+
Salicin	+	Citrate utilization	+
Sorbose	+		

+ = utilized; – = not utilized.

Table 2. Effect of enzymes on activity of the CFS from *Lb. plantarum* M4 against *Staph. aureus* ATCC 43306 (Inhibition zone diameters in mm).

Enzymes	Inhibition zone diameter (mm)
Proteinase-K	0.0
Pepsin	0.0
α-amylase	14
uninoculated medium with no enzyme	0.0
Enzyme-free cell supernatants	14

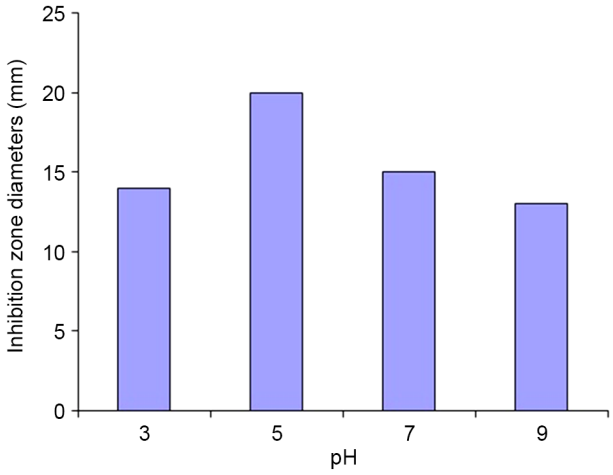

Figure 1. Effect of pH on inhibitory activity of CFS from *Lb. plantarum* M4 on *Staph. aureus* ATCC43306.

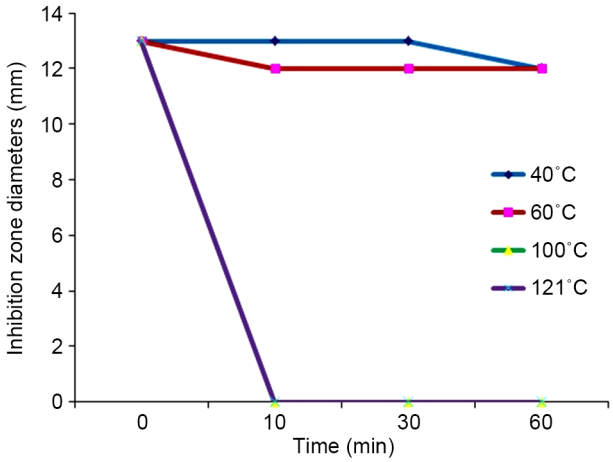

Figure 2. Effect of heating on activity of CFS from *Lb. plantarum* M4.

No drop in activity of the CFS was observed on storage at −80˚C or −20˚C up to four weeks. However, at 4˚C and 35˚C a gradual decline in activity was observed starting from the second week, and by the fourth week, the retained activities were 78.6% and 77% of the starting activities at 4˚C and 35˚C, respectively (**Figure 3**). Bacteriocins produced by *L. plantarum* F1 remained fully stable after storage for 60 days at −20˚C, but declined or became undetectable after storage for 80 to 120 days at 37˚C, indicating that cold temperature may be the most appropriate preservation technique [45].

Figure 4 shows bands produced by the electrophoretic separation of the protein in the *Lb. plantarum* PM4 CFS in comparison to a marker of standard protein molecular weights. The molecular weight of the protein was in the range of 3 - 4 KDa. This is the same as bacteriocin ST414BZ (3.7 kDa) from *Lb. plantarum* ST414BZ [46], plantaricin 35d (4.5 kDa) produced by *Lb. plantarum* 35d [47], bacteriocins ST28MS and ST26MS with 5.5 and 2.8 kDa, respectively [48] and bacteriocin BM-1 of 4638.142 Da [49]. This is within the range of most bacteriocins reported for the genus *Lactobacillus* [40].

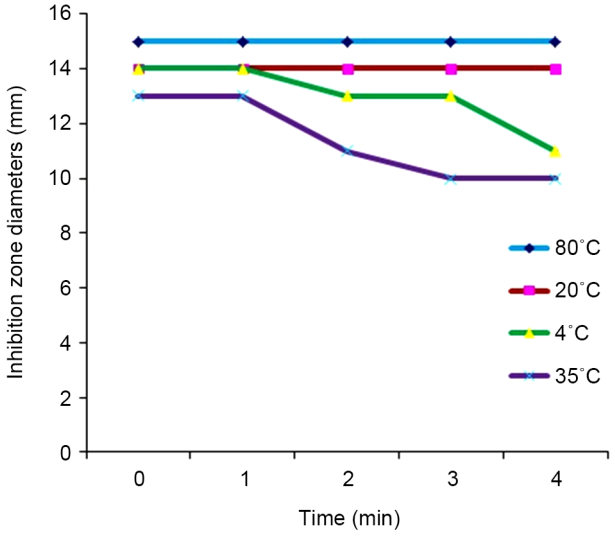

Figure 3. Effect of storage at −80˚C, -20˚C, 4˚C and 35˚C for four weeks on activity of CFS from *Lb. plantarum* M4.

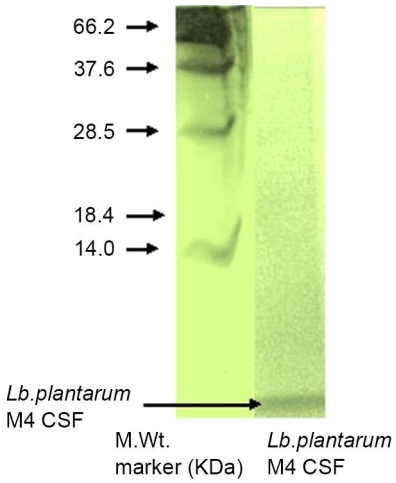

Figure 4. Gel electrophoresis (SDS-PAGE) of the inhibitory moiety of *Lb. plantarum* M4 stained with Coomassie blue.

3.3. Mode of Action

On addition of the CFS from *Lb. plantarum* PM4 to *Staphylococcus aureus* ATCC 43306, growth was completely arrested, with no increase in the optical density of the treated broth culture up to five hours from the time of addition, while the optical density of the untreated broth culture rose from 0.1 to 0.39 during those five hours (**Figure 5**). No growth was obtained on re-culturing the treated broth culture on fresh Nutrient Agar medium indicating that it has a bactericide effect on *S. aureus*. This is similar to the bacteriocidal mode of activity of bacteriocin AMA-K from *Lb. plantarum* AMA-K [50] and plantaricin 35d produced by *Lb. plantarum* 35d [47].

3.4. Spectrum of Activity

Table 3 depicts the spectrum of activity of the CFS against eight bacterial strains.

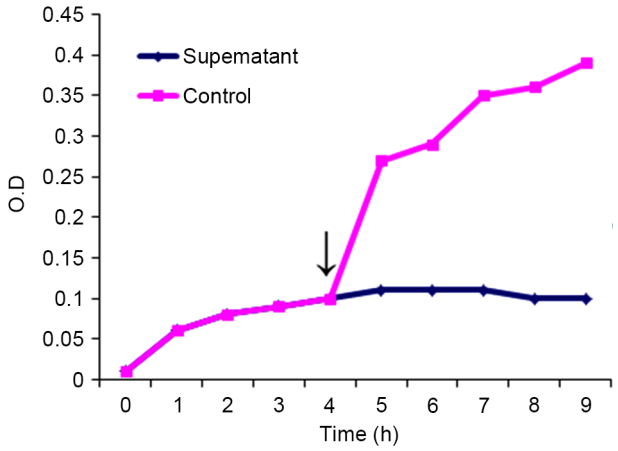

Figure 5. Mode action of CFS from *Lb. plantarum* M4. Arrow indicates time of addition of CFS.

Table 3. Spectrum of inhibitory activity of CFS from *Lb. plantarum* M4 against eight target organisms (zone diameters, mm).

Target organism	Inhibition zone diameter (mm)
Enterococcus faecalis ATCC 10541	12
Staph. aureus ATCC 25923	12
Klebsiella pneumoniae ATCC 10031	9
Proteus vulgaris ATCC 6380	11
Salmonella typhi ATCC 1319106	0
E. coli ATCC 25922	10
E. coli (local isolate)	0
Pseudomonas aeruginosa ATCC 27853	0

It was active against both Gram-positive and Gram-negative bacteria. This spectrum of activity is similar to that reported for other plantaricins. For instance, it was reported that the bacteriocin produced by *Lb. plantarum* was effective against both gram positive and gram negative bacteria [51]. Also bacteriocin C8 from *Lb. plantarum* was reported to have inhibitory activity against not only many Gram-positive but also Gram-negative bacteria such as *Escherichia coli* [39]. *Lb. plantarum* BM-1 isolated from a traditionally fermented Chinese meat was found to produce a novel bacteriocin that is active against a wide range of gram-positive and gram-negative bacteria [49].

3.5. Kinetics of Growth and Bacteriocin Production

Bacteriocin production (measured as inhibitory activity (AU)) by *Lb. plantarum* PM4 generally coincided with the phase of maximum growth (**Figure 6**). The acivity was detected after just 5 h indicating that the bacteriocin is a primary metabolite. However, maximum activity was obtained between 10 and 26 h and maximal growth occurred at the 25 h from inoculation. Similarly, it was reported

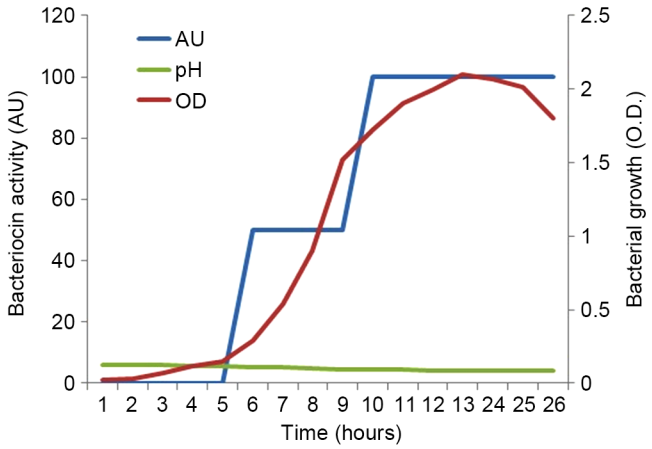

Figure 6. Kinetics of growth and bacteriocin production by *Lb. plantarum* M4.

[48] that two bacteriocins from *Lb. plantarum* (ST28MS and ST26MS) showed detectable levels of inhibitory activity after 5 h suggesting that the peptide is a primary metabolite. Similar results were also reported for plantaricin Y [52] and bacteriocin ST13BR [53]. Both maximum growth and inhibitory activity were obtained at pH 4.4 - 4.2. Bacteriocin production is usually observed to be proportional to growth [54] [55].

3.6. Optimization of Bacteriocin Production

Optimization of bacteriocin production by *Lb. plantarum* PM4 was studied in MRS broth using temperature, pH and length of incubation period (time) as variables. At pH 5.0, production of the inhibitory activity in the CFS was low (around 50 AU/mL) whether the CFS was incubated at 25˚C or 30˚C for up to 72 h. However, at 37˚C, production surged to 100 AU/mL at the first 24 h but then declined to around 50 AU/mL (**Figure 7(a)**). Production at pH 5.5 was generally higher than at pH 5.0, being around 100 AU/mL at the first 24 h, and the surging to 200 AU/mL at 48 h, then receding (**Figure 7(b)**). At pH 6.0, incubation at 25˚C for 48 h resulted in 100 AU/mL while all other incubation conditions resulted in lower yield (**Figure 7(c)**). Accordingly, the best combination of incubation conditions for production of the inhibitory activity by *Lb. plantarum* M4 appears to be at pH 5.5 for 48 h whether incubated at 25˚C, 30˚C or 37˚C.

Maximum production of bacteriocin ST13BR by *Lb. plantarum* ST13BR was recorded at 30˚C and not at 37˚C [53]. However, optimum production of bacteriocin by *Lb. plantarum* F12 was reported at 37˚C [56]. Optimal bacteriocin production (12,800 AU/mL) from *Lb. plantarum* AMA-K was recorded in MRS broth with an initial pH of 6.0 and 5.5 [50]. Also, maximum activity of bacteriocin ST26MS was recorded in MRS broth with an initial pH of 5.5 [48]. It should be noted that the titers reported for CFS are usually thousands of times lower than those reported for purified bacteriocins [57]. The use of bacteria isolated from meat may contribute to a better sensory quality of the meat fermented products [58].

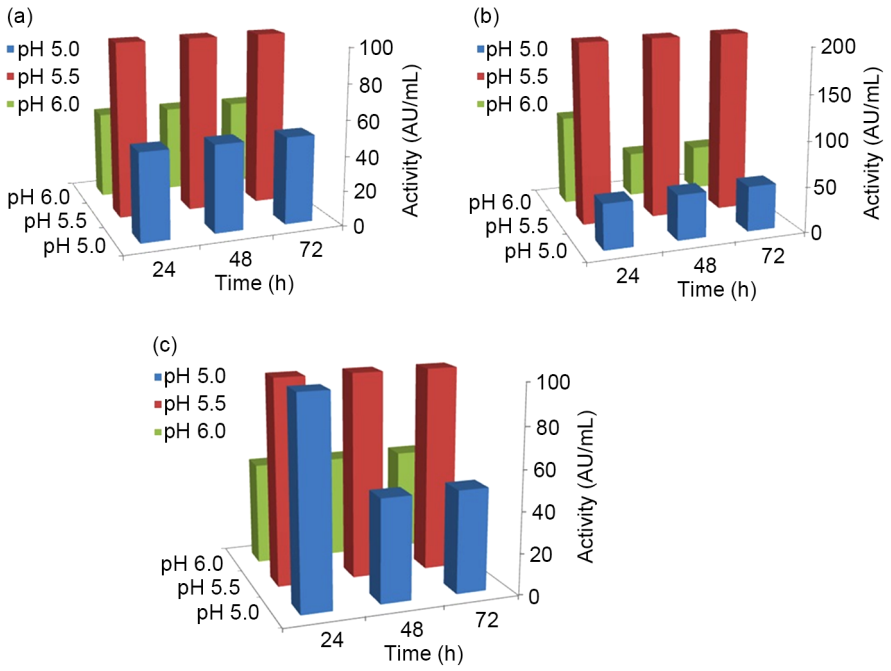

Figure 7. Activity of CFS from *Lb. plantarum* M4 as affected by pH and incubation period at (a) 25˚C, (b) 30˚C and (c) 37˚C.

4. Conclusion

Results of this study indicated that *Lb. plantarum* PM4 isolated from fermented beef (shermout) exhibited promising antimicrobial activity against both Gram positive and Gram negative bacteria, and could be used as a starter culture in the processing of fermented meat as well as biopreservative. Acidification carried out by LAB such as *Lb. plantarum* and the production of bacteriocins contribute, in addition to good manufacturing practices, to the inhibition of food pathogens such as *Salmonella*, *E. coli and S. aureus* and can ensure safe and improved product quality.

Conflict of Interests

The authors declare no conflict of interests whatsoever.

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