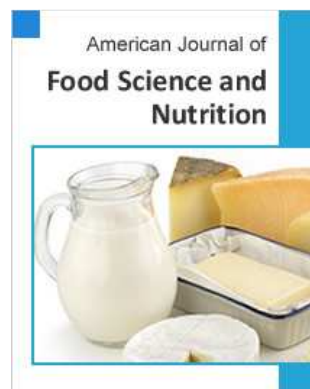




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Bacteriocins (From *Bifidobacterium* spp) Biopreservative Against Gram-Negative Pathogenic Bacteria in Minced Meat as a Critical Control Point

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Abstract

Antimicrobial compounds produced by some bifidobacterium strains (*Bifidobacterium bifidum* and *Bifidobacterium lactis* Bb-12) were subjected to antimicrobial activity towards gram-negative pathogenic bacteria. Agar diffusion method and minimum inhibitory concentration were performed. Bifidobacteria were found to exert strong inhibitory activity towards gram-negative indicator bacteria, namely *Aeromonas hydrophilia*, *Aeromonas caviae*, *Aeromonas sobria*, *Pseudomonas flourescent*, *Pseudomonas aeruginosa*, *Pseudomonas fragi* and *Escherichia coli* 0157:H7. The data showed that *Aeromonas caviae* and *Aeromonas sobria* were more sensitive to bacteriocins than *Aeromonas hydrophilia*, also *Aeromonas* spp. were more resistance to bacteriocins than *Pseudomonas* spp. and *E. coli* 0157:H7. This antimicrobial substance remained active after storage at 3 and -18°C for 3 months, was stable at pH values of 5 to 7 and resistant to heat and showed a bactericidal action. Addition of bifidin or bifilact Bb-12 to ground beef resulted in reduction of *E. coli* count by ratio 100% in sample containing 8% fat when grilled to an internal temperature of 68°C. The results of this study will be beneficial to the food industry in designing HACCP plans to effectively eliminate gram-negative pathogenic bacteria especial *E. coli* 0157:H7 in meat product.

1. Introduction

Application of bacteriocins in food preservation may be beneficial in several aspects [28]: (1) to decrease the risks of food poisoning, (2) to decrease cross-contamination in the food chain, (3) to improve the shelf life of food products, (4) to protect food during temperature-abuse episodes, (5) to decrease economic losses due to food spoilage, (6) to reduce the levels of added chemical preservatives, (7) to reduce the intensity of physical treatments, thereby achieving a better preservation of the food nutritional value and possibly decrease processing cost, (8) possibly to provide alternative preservation barriers for “novel food” and possibly satisfy the demands of consumers for foods with fresh-tasting, lightly preserved, and ready to eat.

Even if the safety of *lactobacilli* and *bifidobacteria* is well documented [28], the use of appropriate in vitro assays to assess the safety of probiotics should not be precluded [53].

L. salivarius CECT 5713 fulfills safety criteria for probiotic bacteria [46] and it has been demonstrated to be safe in animal models [42, 48]. The behavior of this strain is similar to the results previously obtained by [35] using the mixture of the potential probiotic *L. salivarius* W24 and another probiotic Lactobacillus strains (*L. plantarum* DSM 2648, *L. casei* W56, *L. salivarius* W24, *L. acidophilus* W70, *Lactococcus lactis* W58, *Bifidobacterium infantis* W52 or *Bifidobacterium bifidum* W23).

Several mechanisms have been suggested for the inhibitory action of bifidobacteria towards Gram-negative pathogens, including a decrease of the local pH by the production of organic acids, the inhibitory action of undissociated organic acid molecules, the competition for nutrients, the competition for adhesion sites, the stimulation of the host's immunity, and the production of specific antibacterial substances [6, 19, 25]. Organic acids, in particular acetic and lactic acids, have a strong inhibitory effect against Gram-negative bacteria, however, only few authors suggested that the production of organic acids is the sole factor responsible for the antagonistic activity of bifidobacteria [23, 24, 31]. In numerous reports it has been suggested that other inhibitory substances may contribute to the antagonistic activity as well [54].

The objectives of this research were to examine some bifidobacterium strains for possible production of bacteriocins and to determine the antimicrobial activity of these bacteriocins against some gram-negative pathogenic bacteria. We also assessed effect of bacteriocin on the change in heat resistance of *E. coli* 0157:H7 in nutrient medium and minced meat.

2. Materials and Methods

2.1. Materials

Cow meat (round cuts) and fat were purchased from a local market in Ismailia, Egypt. The meat was cut into small pieces and frozen at -18°C for 12 hrs. Frozen meat and fat were minced two times (using small house mincer) then divided into two portions (8 or 15% fat) and used for experiments. Fat content was determined according to [5].

All reagents were analytical grade and purchased from Top Quality Co. (Sigma chemical Co.) All media used in microbiological analysis; Starch Ampicillin Agar (SAA) for enumerate *Aeromonas spp.*, Pseudomonas Agar for enumerate *Pseudomonas spp.*, Tryptic Soy Agar (TSA) for enumerate *E. coli* 0157:H7 and (Man, Rogosa and Sharpe (MRS) for enumerate *Bifidobacterium spp.*, were obtained from Top Quality Co. (Merck - Germany). *Bifidobacterium bifidum* and *bifidobacterium lactis* (Bb-12) were originally obtained from DVS, Chr Hansen's lab. Denmark. Test cultures of pathogenic organisms *Aeromonas spp* (*A. hydrophilia*, *A. caviae*, *A. sobria*) and *Pseudomonas spp* (*P. fluorescent*, *P. aeruginosa*, *P. fragi*) were obtained from Department of Food Hygiene, Animal Health Research Institute, Cairo, Egypt.

Cultures were transferred separately from one-day-old

cultures into fresh nutrient broth medium which were incubated at 26°C for 24 hrs before inoculation [58]. *E. coli* 0157:H7 (ATCC 69373) strain was obtained from Cairo Microbiological Resource Center (MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Stock culture was maintained on TSA (Biolife), slants stored at 3°C. Every 7 to 10 days, a loopful of this culture was transferred to fresh TSA slants and incubated for 24 hrs at 37°C. These cultures were then stored at ambient temperature and used as stock cultures for experimental procedures [44]. For inoculation, cultures were transferred to Tryptic Soy Broth (TSB) (Biolife) and grown at 37°C for 12 hrs, cultures were then serially diluted in 0.1% peptone and inoculated into fresh TSB, followed by incubation at 37°C for 12 hrs for growth to stationary phase. Stationary phase cultures were used since such cultures have been shown to have greater heat resistance than log-phase cultures [58].

2.2. Methods

Preparation of crude bacteriocin:-

B. bifidum or *B. lactis* Bb-12 were inoculated into MRS broth at 1% (vol/vol). After 16 hrs of incubation at 37°C, the culture supernatant of each was collected by centrifugation at 22100 × g for 30 min. The supernatant was sterilized by filtration through 0.45 and 0.22 µm-pore-size filters and precipitated with 70% ammonium sulfate. The precipitated was dissolved in deionized water. The resulting solution was designated crude bacteriocin.

a) Antibacterial activity of bacteriocin:

The crook borer diffusion method was used to measure the antibacterial activity according to the method described by [43] by removing a slug from solidified agar with a sterile crook borer (1 cm diameter) after inoculation with bacteria. Bifidocin were added at concentration of 50, 100 and 150 µl in duplicates.

Inoculated plates were incubated at 26°C for *Pseudomonas* and *Aeromonas* sp. and 37°C for *E. coli* 0157, and the inhibition zones of the microbial growth produced by different bifidocin were measured.

b) Minimum Inhibitory Concentration (MIC) of bacteriocin:

Minimum Inhibitory Concentration (MIC) determination was carried out according to the method described by [7] with modification as following:

One ml of 24 hrs-activated cultures was serially diluted and one ml from each dilutions (in duplicates) was transferred into petri dishes. Different concentrations of bacteriocin from 20 µl up to 160 µl were thoroughly mixed with sterilized nutrient agar (10 ml), then poured into aforementioned petri dishes. The dishes were incubated at 26°C for *Pseudomonas* and *Aeromonas* sp. and 37°C for *E. coli* 0157. The viable colonies were counted, and inhibition percent was calculated and referred to the viable count at 0.0% bacteriocin.

c) Sensitivity of bacteriocin to heat:

Five millilitres of the concentrates obtained from potential bacteriocin- producing strain cultures, were treated separately

in a water bath at 60, 80 and 90 °C for 1/2 hr; also in an autoclave (Autotester G- dry, Selecta, Spain) at 115 °C for 15 min. Immediately, samples were cooled to 4 °C and the residual activity determined by the well diffusion assay. Untreated samples were included as controls.

d) Sensitivity of bacteriocin to pH:

The concentrates were dispensed in tubes and the pH was adjusted by lactic acid (1% w/v) or (1 M) NaOH in the range was 4.0-9.0. After 2 hrs at room temperature, pH was readjusted to 7.0. The residual activity was determined by the well diffusion assay. The concentrates of unadjusted pH were used as the controls.

e) Stability of bacteriocin during storage:

To test stability during the storage, the concentrate was stored at 25, 4 and -18 °C for 3 month. The residual activity determined by the well diffusion assay.

2.3. Control of *E. coli* 0157:H7 by Bacteriocin as Critical Control Point

A) Thermal resistance of *E. coli* in a nutrient medium:

0.4 ml of stationary phase culture, prepared as described above, was inoculated into tubes containing 3.6 ml of sterile TSB plus 120 µl bifidin or bifilact, tube without bacteriocin recorded as control. Tubes were immersed in constant temperature water bath at 55 ±1° C. temperature was monitored using a hand held digital thermometer (inserted into sealed vials filled with 4 ml of TSB). When sample vials reached 55°C, three tubes were removed and placed in an ice water bath for 30 s., these samples represented the initial (0 min.) population for the determination of heat resistance at 55°C. Three tubes were then removed from the water bath at 5 min for up to 30 min., cooled vials were stored at 3°C prior to analysis. Enumeration of surviving organisms in each sample was performed by serial dilutions of cultures and inoculation onto TSA using a spread plate technique. Plates were incubated at 37°C for 24 hrs prior to counting. Log CFU/ml survivor curves at 55°C were determined from the survival data obtained [34].

Study the hold stress on thermal resistance of E. coli:

To evaluate changes in thermal resistance of *E. coli* as a result of hold stress (with 120-µl bacteriocin) at 25°C, inoculated vials were subjected to holding at 25 ±1°C for 3, 6 and 9 hrs prior to heating at 55°C. Survivor curve at 55° C for the samples treated were determined as described.

Study the salt stress on thermal resistance of E. coli:

To evaluate changes in thermal resistance of *E. coli* as a result of salt stress (with 120-µl bacteriocin). Stationary-phase cultures were inoculated into vials supplemented with 1.0, 1.5 and 2.0 % sodium chloride and the heat resistance at 55°C was determined as previously described.

Study the cold and freeze stress on thermal resistance of E. coli:

Samples (inoculated vials containing 120-µl bacteriocin) were frozen at -18 ±1°C or refrigerated at 5 ±1°C for seven days. After storage period, tubes from storage treatments were immersed in constant temperature water bath at 55 ±1°C. Frozen and refrigerated samples were thawed to room

temperature prior to heating. The heat resistance at 55°C was determined as previously described.

B) Thermal resistance of *E. coli* in minced meat:

One or two ml of inoculate per/100g of minced meat was hand mixed with minced meat. The hand mix process was performed with sterile glass rod for about two minutes to ensure that inoculants and minced meat were well mixed. The final population was 10⁶ CFU/g of meat [45].

Three different treatments of every portions (8 or 15% fat) were achieved by the addition of bacteriocin (bifidin or bifilact) to give a final concentration 0 (control, receiving no bacteriocin), 1.2 % bifidin and 1.2 % bifilact. Every treatment divided into portions (50 g) were weighted into sterile pouches. Thereafter, the bags were manually mixed to ensure even distribution of the organisms in the meat sample, compressed into a thin layer (Ca, 1.0 cm thick) by pressing against a flat surface excluding most of the air [14].

Duplicate patties from each portion were grilled (after 2 or 3 hrs from adding bacteriocin) to an internal temperature of 62°C (to approximate a medium degree doneness), or 68°C (to approximate a medium- well to well - done degree of doneness). Temperature was monitored using a hand held digital thermometer by placing into center of each patty and inserting an additional thermocouple into various locations in each patty during cooking to determine the location of the minimum internal temperature. This location was used to determine when the desired patty end-point temperature had been reached. When cooking to a target internal temperature of 62°C, the patties were turned over when the internal temperature reached 31°C and immediately removed from the grill when the internal temperature reached the targeted 62°C. likewise, when cooking to the target internal temperature of 68°C, the patties were turned over at 34°C internal temperature and immediately removed from the grill when the internal temperature reached 68° C. Cooking time (min) was recorded for each patty. Each cooked patty was transferred to an individual sterile petri dish for analysis. [22].

Statistical analysis:

Standard deviation (SD) and significant differences between the mean values of the estimated tests were performed using the software package Statistical 9.1 for Windows, Stat Soft, Tulsa, Oklahoma, USA, 2009. Differences were considered significant at P < 0.05.

3. Results and Discussion

3.1. Antimicrobial Activity of Bacteriocin

Preliminary screening of the antibacterial activity in vitro of the bacteriocin from bifidobacteria (*B. bifidum*, *B. lactis* Bb-12) was studied against some gram-negative bacteria (*Aeromonas hydrophilia*, *caviae*, *sobria*, *Pseudomonas flourescent*, *aeruginosa*, *fragi* and *E. coli* 0157:H7), using the diffusion method.

The data tabulated in Table (1) for inhibition zones (mm) of various microorganisms indicated that bifidin had moderate effect against gram-negative bacteria whereas; bifilact Bb-12

was highly active. These data agreed with those obtained by [49] who reported that twelve strains of bifidobacterium, had a broad spectrum of antagonistic activity against both gram-positive and gram-negative indicators, especially *Pseudomonas* species. *Bifidobacterium animalis* 31 and *B. breve* J were reported to produce antimicrobial compounds

having inhibition effects towards *Salmonella enteritidis* 9 J [10]. Certain bifidobacteria also have inhibition activity against *E. coli* 0157:H7 [27] and substances other than organic acids obtained from *B. longum* and *B. infantis* have also been reported to inhibit *B. cereus* and *E. coli* [17].

Table (1). Antimicrobial spectrum of bacteriocin (Mean $\pm$ standard deviation).

Species	Diameter of inhibition zone (mm)					
	Bifidin conc.			Bifilact Bb12 conc.		
	50 μ l	100 μ l	150 μ l	50 μ l	100 μ l	150 μ l
<i>Aeromonas hydrophilia</i>	7 $\pm$ 0.5	11 $\pm$ 0.8	21 $\pm$ 0.2	8 $\pm$ 0.5	18 $\pm$ 0.1	25 $\pm$ 0.2
<i>Aeromonas caviae</i>	8 $\pm$ 0.4	14 $\pm$ 0.1	25 $\pm$ 0.5	10 $\pm$ 0.4	16 $\pm$ 0.1	31 $\pm$ 0.5
<i>Aeromonas sobria</i>	7 $\pm$ 0.4	12 $\pm$ 0.4	23 $\pm$ 0.1	9 $\pm$ 0.1	21 $\pm$ 0.3	27 $\pm$ 0.3
<i>Pseudomonas fluorescens</i>	10 $\pm$ 0.1	18 $\pm$ 0.3	23 $\pm$ 0.3	11 $\pm$ 0.1	19 $\pm$ 0.2	26 $\pm$ 0.1
<i>Pseudomonas aeruginosa</i>	11 $\pm$ 0.8	19 $\pm$ 0.1	24 $\pm$ 0.3	12 $\pm$ 0.5	18 $\pm$ 0.6	28 $\pm$ 0.4
<i>Pseudomonas fragi</i>	8 $\pm$ 0.5	17 $\pm$ 0.8	23 $\pm$ 0.1	9 $\pm$ 0.7	18 $\pm$ 0.1	26 $\pm$ 0.2
<i>E. coli</i> 0157:H7	10 $\pm$ 0.1	18 $\pm$ 0.5	25 $\pm$ 0.7	11 $\pm$ 0.1	19 $\pm$ 0.3	28 $\pm$ 0.2

3.2. Minimum Inhibitory Concentrations (MIC)

Minimum inhibitory concentrations of bifidin and bifilact against various species of *Aeromonas* and *pseudomonas* bacteria are shown in Table (2). The data showed that *eromonas* spp. were more resistance to bacteriocin (bifidin or

bifilact) than *Pseudomonas* spp. generally, the reduction of viable bacterial cells gradually increased according to increasing bacteriocin concentrations which were added to the growth agar medium.

Table (2). Minimum inhibitory concentrations of bacteriocin on some *Aeromonas* spp., *Pseudomonas* spp. and *E. coli* 0157:H7.

Microorganisms	Bacteriocin concentration (μ l)								
	0	20	40	60	80	100	120	140	160
Bifidin (Inhibition %)									
<i>Aeromonas hydrophilia</i>	0.0	2.11	10.51	24.37	46.82	58.95	79.62	92.14	100.0
<i>Aeromonas caviae</i>	0.0	5.63	18.23	36.11	55.22	71.13	93.18	100.0	—
<i>Aeromonas sobria</i>	0.0	3.21	13.91	28.17	53.14	70.22	91.16	100.0	—
<i>Pseudomonas fluorescens</i>	0.0	4.21	15.60	24.13	47.21	60.11	81.25	100.0	—
<i>Pseudomonas aeruginosa</i>	0.0	6.13	21.72	37.91	57.32	74.18	95.73	100.0	—
<i>Pseudomonas fragi</i>	0.0	4.32	16.11	28.19	52.19	71.22	93.68	100.0	—
<i>E. coli</i> 0157:H7	0.0	7.89	37.20	61.31	91.00	100.0	—	—	—
Bifilact (Inhibition %)									
<i>Aeromonas hydrophilia</i>	0.0	5.19	21.53	59.17	75.31	93.8	100.0	—	—
<i>Aeromonas caviae</i>	0.0	11.28	29.78	62.19	89.11	100.0	—	—	—
<i>Aeromonas sobria</i>	0.0	7.39	27.11	60.32	87.20	100.0	—	—	—
<i>Pseudomonas fluorescens</i>	0.0	9.81	31.14	51.13	84.18	100.0	—	—	—
<i>Pseudomonas aeruginosa</i>	0.0	15.22	37.63	66.71	91.22	100.0	—	—	—
<i>Pseudomonas fragi</i>	0.0	8.99	33.16	59.32	86.41	100.0	—	—	—
<i>E. coli</i> 0157:H7	0.0	8.11	55.91	63.12	87.81	100.0	—	—	—

The tabulated results indicated that *Aeromonas cavia* and *Aeromonas sobria* were more sensitive to bacteriocin than *Aeromonas hydrophilia*, which were completely inhibited in presence of 1.6% bifidin and 1.2% bifilact, while *Aeromonas hydrophilia* was still viable when the same concentrations were added to the growth agar media. On the contrary, *Pseudomonas* spp. were inhibited when 1.4% of bifidin and 1.0% bifilact were added. From the same table it can be noticed that *E. coli* 0157:H7 was inhibited with concentration of 1.0% of bifidin or bifilact.

The expected action of bacteriocin against gram-negative bacteria can be attributed to compounds (acetic and lactic acids) and possibly other antimicrobial substances produced during culturing. Similar results were obtained by [16] who reported that certain bifidobacterium strains are able to

produce substance that compete and prevent pathogenic bacteria from adhering to the receptors on epithelial cells of intestinal surfaces.

Bifidobacteria have the ability to produce organic acids and other antimicrobial compounds such as proteinaceous compounds called bacteriocins [30, 32, 62, 63]. Some studies have attributed the antimicrobial activity of bifidobacteria solely to their pH-reducing effects [13, 31] as a result of their production of lactic and acetic acid, while others have attributed it to the production of proteinaceous compounds in addition to the pH reducing effects [4, 29, 47]. Also, [64] reported that different food borne pathogens like *Escherichia coli*, *Listeria* spp., *Salmonella* spp., *Staphylococcus aureus*, *Aeromonas hydrophilia*, *Vibrio anguillarum*, and *Bacillus cereus* were inhibited by cell-free supernatants produced by

lactobacilli isolated from Sturgeon fish.

The data in Table (3) show that bacteriocin (bifidin or bifilact Bb-12) activity after exposure to pH values of 4 to 10 after 2hrs at room temperature (25°C), was stable at pH levels

between 5 to 7. However, its activity was decreased at pH 4, 8 and 9. Bifilact Bb-12 was relatively more stable at acidic than basic conditions.

Table (3). Effect of different treatment on bacteriocin activity (Mean $\pm$ standard deviation).

Treatment	Inhibition zone (mm)	
	bifidin	bifilact
*Control	11 $\pm$ 0.5	13 $\pm$ 0.1
Heating		
60° C, 1 h	11 $\pm$ 0.4	13 $\pm$ 0.8
80° C, 1 h	11 $\pm$ 0.5	13 $\pm$ 0.6
90° C, 1 h	11 $\pm$ 0.3	13 $\pm$ 0.3
115° C, 15 min	11 $\pm$ 0.2	13 $\pm$ 0.4
pH (at 25° C for 2hrs)		
4.0	2 $\pm$ 0.2	5 $\pm$ 0.1
5.0	10 $\pm$ 0.2	10 $\pm$ 0.3
6.0	10 $\pm$ 0.1	10 $\pm$ 0.3
7.0	6 $\pm$ 0.3	7 $\pm$ 0.5
8.0	2 $\pm$ 0.2	2 $\pm$ 0.1
9.0	—	—
Storage		
25° C, 3 month	3 $\pm$ 0.1	5 $\pm$ 0.8
4° C, 3 month	8 $\pm$ 0.1	8 $\pm$ 0.1
-18° C, 3 month	10 $\pm$ 0.3	10 $\pm$ 0.6

*control: without any treatment.

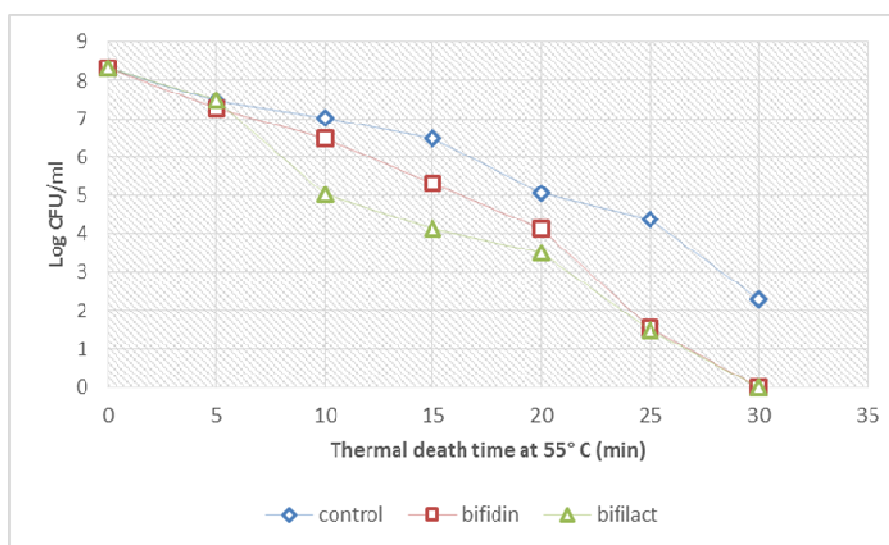


Fig. (1). Survivor curve of *E. coli* 0157:H7 in nutrient medium containing bacteriocin, resulting from heating at 55°C.

The activity of bacteriocin was decreased by 80 and 50% at pH 4 or below and was decreased by 80% at pH 8 or above for bifidin and bifilact Bb-12 respectively. On the other hand, the activity was remarkably stable after heating (60, 80, 90°C for 1hrs and 115°C for 15 min), as no difference was detected in the diameter of the inhibitory zone at any temperature for bifidin or bifilact Bb-12). The bacteriocin was very stable after 3 months of storage at -18°C but its inhibitor activity decreased at room temperature and refrigeration (Table, 3). Similar results reported by [56] showed that bacteriocin substance of *E. faecium* was very stable during 12 months of storage at -20°C but its inhibitor activity slightly decreased at

room temperature and refrigeration. It was found to be stable after heating (100°C for 10 min). [15] reported that bacteriocin produced by *bifidobacterium infantis* showed high temperature stability up to 121°C for 15 min with no loss in its activity and had pH stability in the range of 4-10. The temperature and heat stability of bacteriocin makes it useful for applications in food processing technologies and food safety control applications. Lacticin 3147 is a well characterized bacteriocin that possesses a number of technological favorable features as it is heat stable, active over a broad pH range and has a wide spectrum of activity [65].

3.3. Control of *E. coli* 0157:H7 by Bacteriocin as Critical Control Point

Fig (1) shows that bacteriocin (bifidin or bifilact Bb-12) decreased heat resistance of *E. coli* 0157:H7. This decrease in heat resistance may be due to the antimicrobial activity of compounds produced by *B. bifidum* or *B. lactis* Bb-12 against *E. coli* 0157:H7. [1] reported that bifilact Bb-12 had inhibition activities toward *E. coli*. Certain bifidobacteria also have inhibition activity against *E. coli* 0157:H7 [27]. [41] concluded that *B. bifidum* RBL 71 and *B. bifidum* RBL 460 have been reported to have a significant potential for reducing adhesion of *E. coli* 0157:H7.

The data illustrated in Figs (2, 3, 4) shows that heat resistance for *E. coli* 0157:H7 increased in control sample after 3, 6 and 9 hrs of holding at 25°C comparing with control sample without holding (Fig. 3). This resistance could be a

result of physiological process within the cells or combination of factors including culture age, growth phase or cell density [34]. Also, [59] reported that *P. fluorescens* showed higher resistance at 25°C than at 4°C. Several studies have reported that growth and storage temperatures influence the heat resistance of microorganisms [60]. Such increase in resistance could be an important concern if conditions resulting in this increase were reproduced in a food system. However, if the increase in heat resistance is a result of high population density, it is unlikely that these conditions would occur in food. [9] demonstrated the importance of temperature control during meat handling and storage to prevent the outgrowth of this pathogen and indicated that proper sanitation and processing practices that prevent and reduce contamination of carcasses with *E. coli* 0157:H7 are essential regardless of background microflora levels.

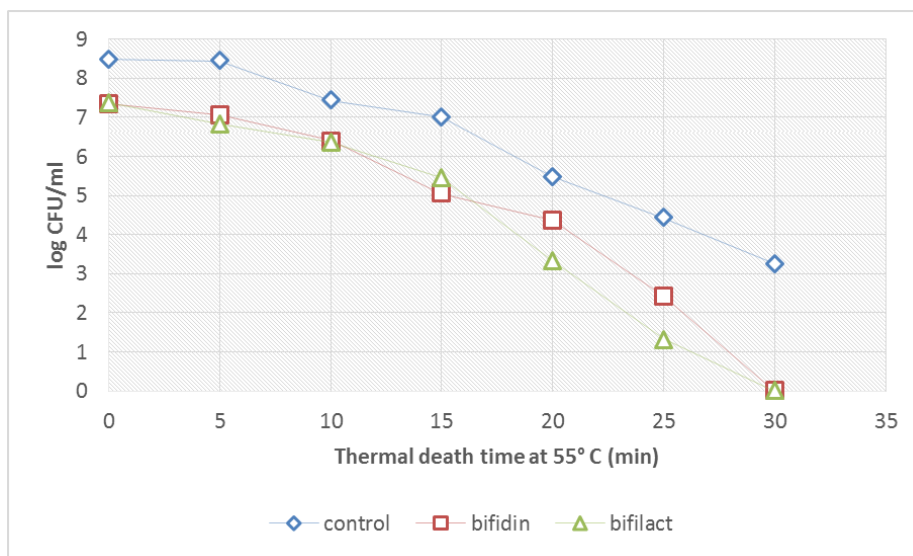


Fig. (2). Survivor curve of *E. coli* 0157:H7 in nutrient medium containing bacteriocin, resulting from heating at 55°C after holding at 25°C for 3 hours.

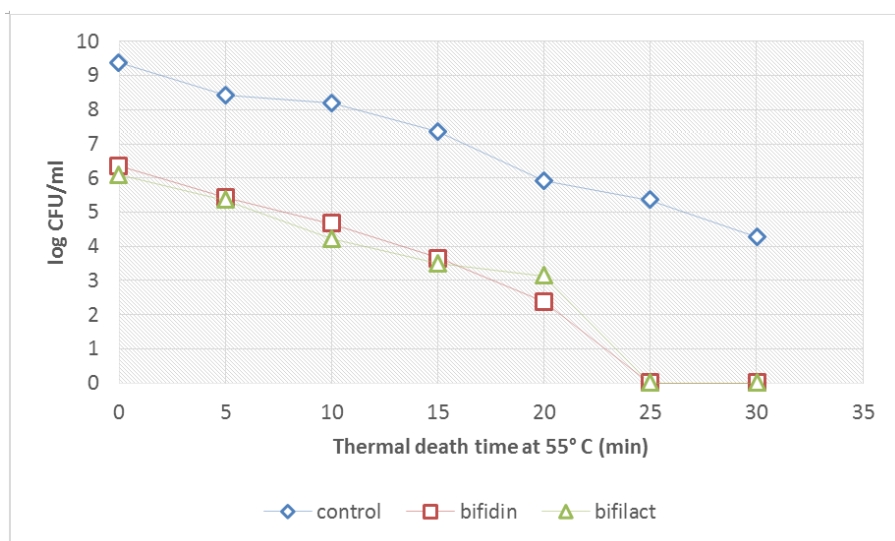


Fig. (3). Survivor curve of *E. coli* 0157:H7 in nutrient medium containing bacteriocin, resulting from heating at 55°C after holding at 25°C for 6 hours.

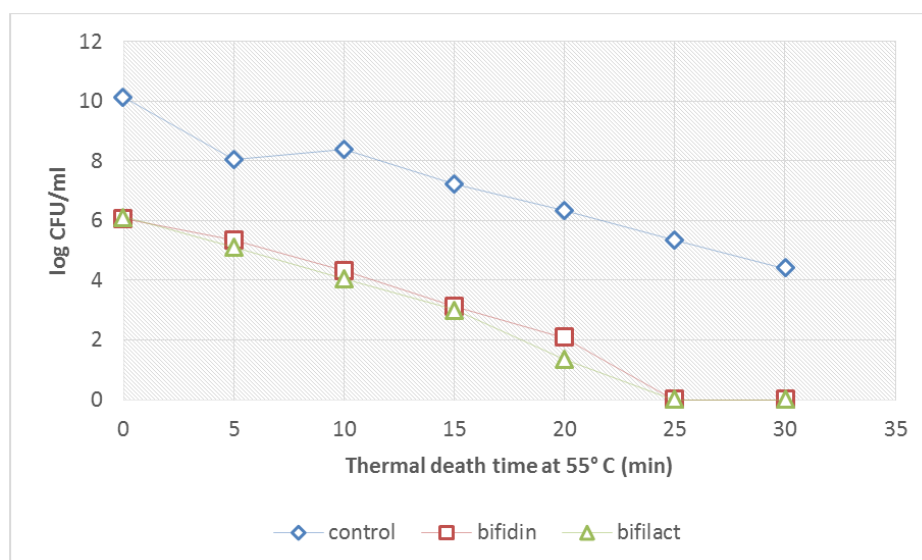


Fig. (4). Survivor curve of *E. coli* 0157:H7 in nutrient medium containing bacteriocin, resulting from heating at 55°C after holding at 25°C for 9 hours.

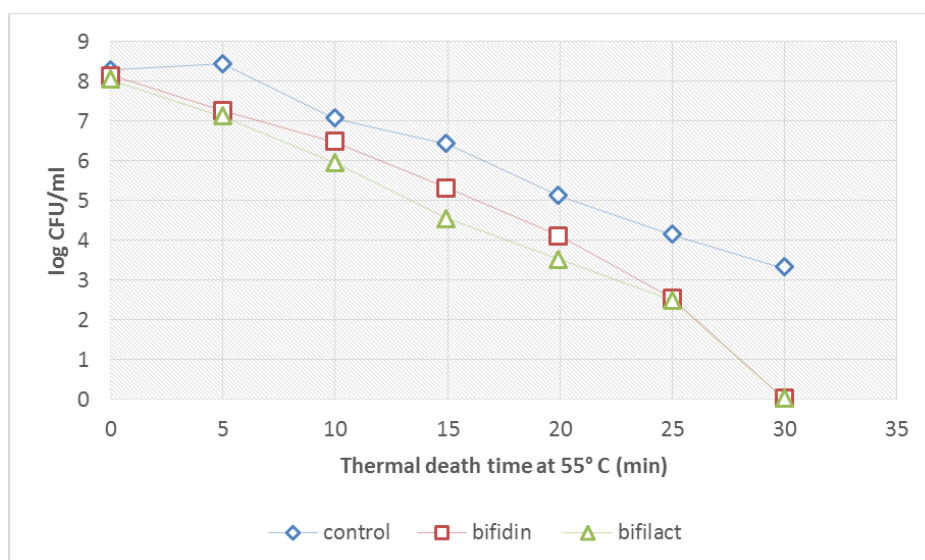


Fig. (5). Survivor curve of *E. coli* 0157:H7 in nutrient medium containing bacteriocin supplemented with 1% sodium chloride, resulting from heating at 55°C.

From the same data it can be noticed that the treated sample with 1.2% bifidin or bifilact Bb-12 showed significant reduction in heat resistance as compared to control after holding for 3, 6 and 9 hrs at 25°C. these reduction might be due to the inhibitory effect of bacteriocin. [16] concluded that several bifidobacteria produce metabolites such as the bacteriocins, which have potent antimicrobial activities towards some of the food-borne pathogens such as *C. perfringens*, *E. coli*, *Salmonella* and other human health threatening pathogens such as the *H. pylori*. The inhibition activity seems to be related to the adhesion ability of bacteriocin onto harmful organisms and thereby inhibiting their growth. Only a very small number of antimicrobial compounds from bifidobacteria have been purified and characterized and therefore there is a potential in further research in this field.

From Figs (5, 6, 7) it can be noticed that heat resistance for

E. coli 0157:H7 increased in all sample after salt stress with 1% sodium chloride comparing with samples without salt. These results are in agreement with those of [37], who reported that a decrease in a_w (from 0.98 to 0.96) resulted in an increase in the heat resistance of *E. coli* 0157:H7 in salt and sucrose solutions. Also, [26] reported that D_{55} values increased from 7.1 in control samples to 8.5 and 8.9 min after salt stress with 0.5 and 1.0% sodium chloride respectively. On the other hand, the heat resistance decreased after salt stress with 1.5 and 2% sodium chloride (Fig 6, 7). This decrease could be a result of a physiological process with in the cell. Such diversity in results has been recognized within a recent review of the published data on the heat resistance of *E. coli* 0157:H7 [55], which noted reported D-values for *E. coli* 0157:H7 in meat at 60°C ranging from 0.3 to 10 min. such variation has been attributed to differences in test conditions and experimental procedures used in the reviewed studies. Factors shown to influence

estimates of heat resistance, leading to inconsistencies between studies, include differences among strains, the physiological conditions of the *E. coli* 0157:H7 examined, the composition of the suspending medium and different thermal inactivation and survivor enumeration methodologies. At the same time addition of bacteriocin (1.2 %) with salt (1.5-2 %) resulted in inhibition of all viable *E. coli* 0157:H7 after 25 min of heat at 55°C, this phenomenon may be due to antimicrobial components production of bifidobacteria include organic acids, hydrogen peroxide, carbon dioxide, diacetyl, bacteriocins and low molecular weight antimicrobial

substances [50]. Similar results observed by [8] who reported that probiotic strains might represent an effective alternative approach to control food-borne enteric pathogens. [66] concluded that a *Lactococcus lactis* subsp. *lactis* strain (*L. lactis* 69) capable to produce a heat-stable bacteriocin was isolated from charqui, a Brazilian fermented, salted and sun-dried meat product. The bacteriocin inhibited, in vitro, *Listeria monocytogenes*, *Staphylococcus aureus*, several lactic acid bacteria isolated from foods and spoilage halotolerant bacteria isolated from charqui.

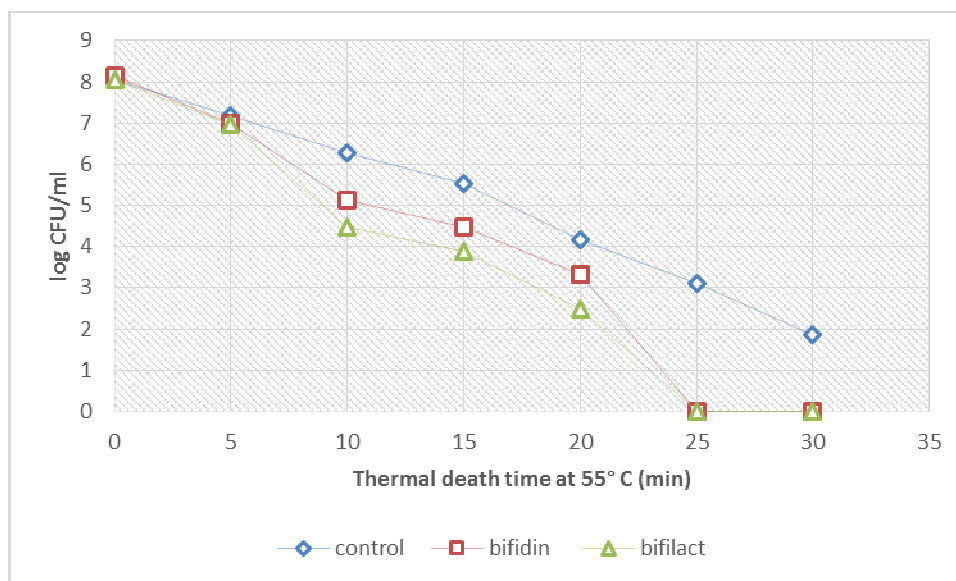


Fig. (6). Survivor curve of *E. coli* 0157:H7 in nutrient medium containing bacteriocin supplemented with 1.5% sodium chloride, resulting from heating at 55°C.

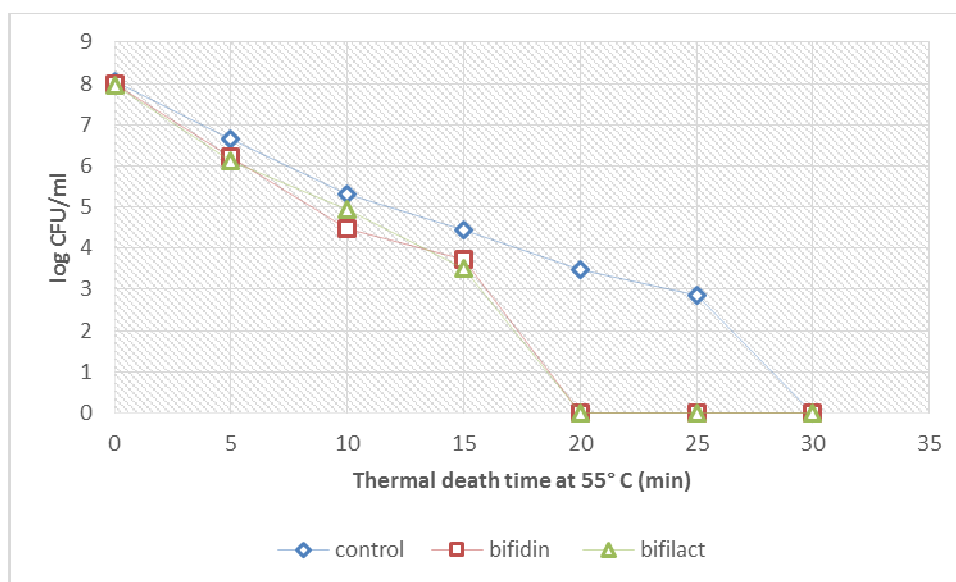


Fig. (7). Survivor curve of *E. coli* 0157:H7 in nutrient medium containing bacteriocin supplemented with 2% sodium chloride, resulting from heating at 55°C.

Fig (8, 9) shows that cold stress decreased the heat resistance of *E. coli* 0157:H7 in control sample and treated samples after cold storage for seven days and sample treated with bifidin or bifilact Bb-12. The decrease in heat resistance following cold stress in control sample may be due to the

induction of cold shock proteins and the repression of heat shock proteins [44]. In the present study, control cultures grown to stationary phase at 37°C were more heat resistance than similar cultures, which had been stored at 4°C prior to heating. [18] reported that several stressing factors including

heat, pH, chemicals or physical agents, induced an elevated resistance to a subsequent stress.

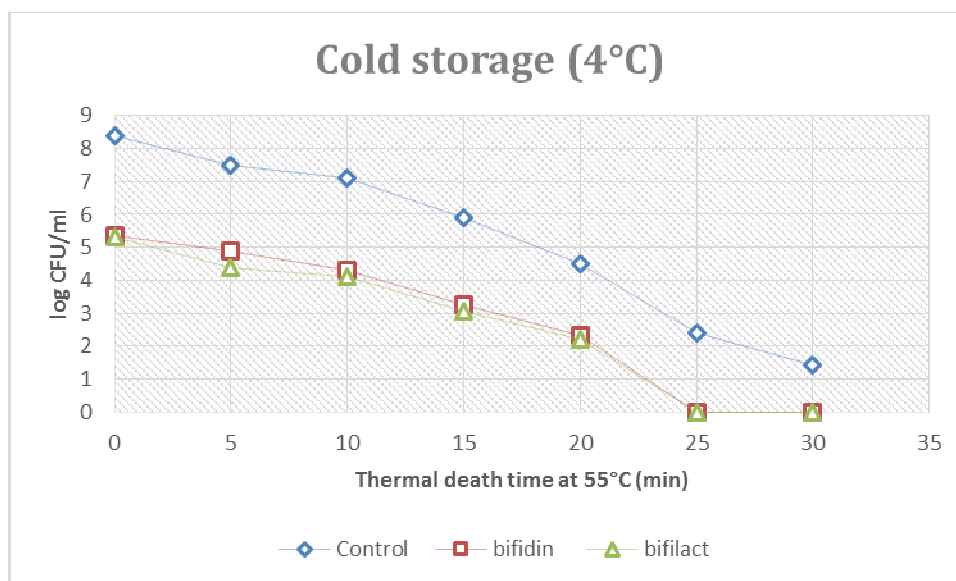


Fig. (8). Survivor curve of *E. coli* 0157:H7 in nutrient medium containing bacteriocin, resulting from heating at 55°C after cold storage at 4°C for seven days.

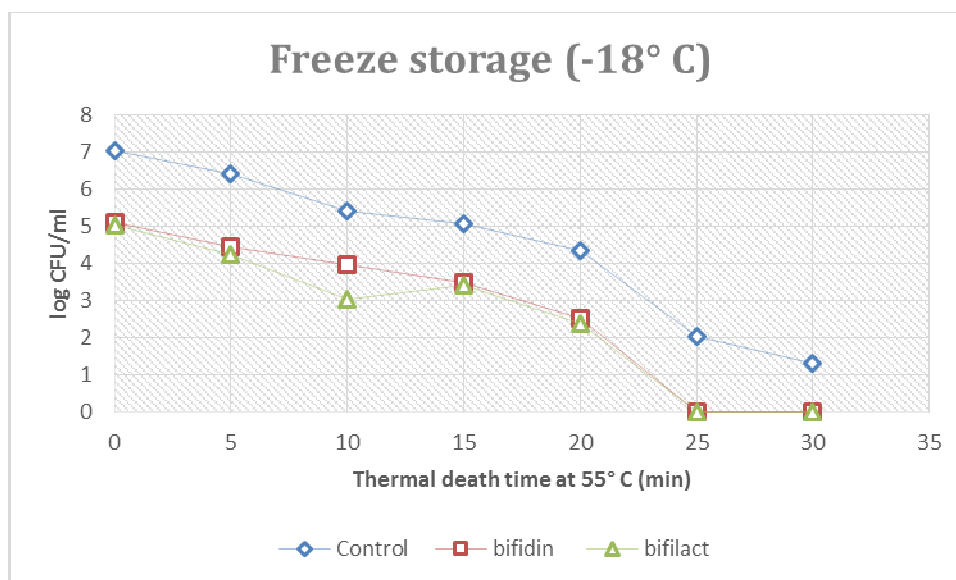


Fig. (9). Survivor curve of *E. coli* 0157:H7 in nutrient medium containing bacteriocin, resulting from heating at 55°C after freeze storage at -18°C for seven days.

The same data shows that all samples (control and treated) stored for seven days at - 18°C appeared more heat sensitive in all samples. The greater heat sensitivity of cultures stored at - 18° C could be a sensitization of cells to the freezing process. These findings are in contrast to the results of [33, 44, 51] who demonstrated that cell membrane flexibility is an important factor in freeze-thaw stress resistance. It is known that membrane flexibility can be modified by the binding of saccharides acting as cryoprotectants or by alteration of the phospholipid and neutral lipid compositions. [34] concluded that the variations in heat resistance could occur due to storage temperatures, growth phase as well as product formulation, sampling techniques and even strain differences within a species or serotype. [16] reported that bacteriocins or

bacteriocin-like compounds produced by bifidobacteria have antimicrobial activities towards harmful microorganisms.

3.4. Effect of Fat Content and Bacteriocin on Heat Resistance of Inoculated Ground Beef

The data tabulated in Table (4, 5) and illustrated in Figs (10, 11), shows that ground beef containing 15% fat offered protection compared to 8% fat, indicating by higher recovery of heated *E. coli* 0157:H7 cells in case of 15% fat by decreasing reduction ratio 34.38 and 48.83 % at 62 and 68°C respectively while these ratio were 49.36 and 58.61% in case of 8% fat (in control sample). Similar results were observed by [36] who reported that the increased thermal resistance of *E.*

coli 0157:H7 in beef compared to chicken may be attributed to the effect of different species and the differences in fat content between the substrates. [2] reported that the D-value of *E. coli* 0157:H7 in ground beef heated at 60°C in thermal death time tubes ranged from 0.45 (beef 7 % fat) to 0.47 (beef, 20% fat)

min; the values ranged from 0.38 min (3 % fat) to 0.55 min (11 % fat) in chicken. Slight differences by previous workers may be attributed to different *E. coli* 0157:H7 strains, methodology, physiological conditions of the cells and fat content [3, 60].

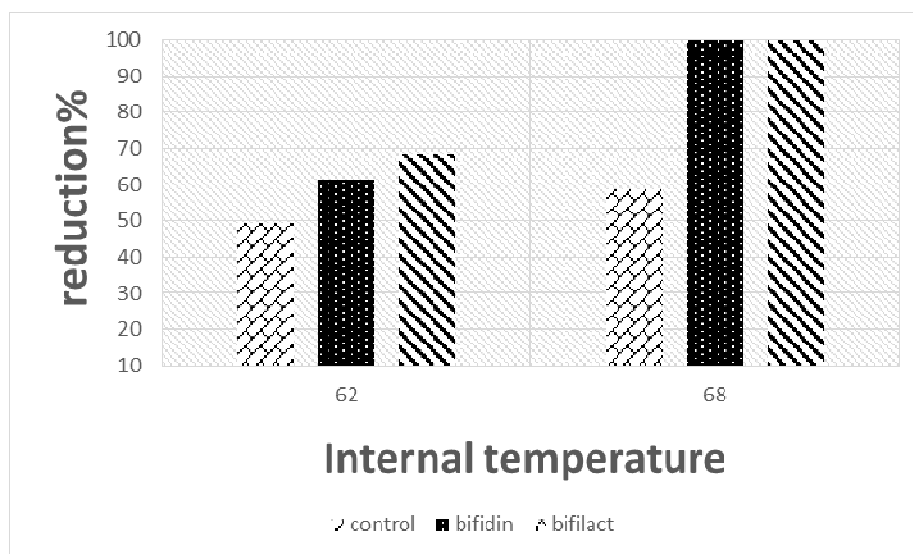


Fig. (10). Reduction ratio of *E. coli* 0157:H7 in ground beef patties (8% fat) treated with bacteriocin and grilled to an internal temperature of 62 or 68°C.

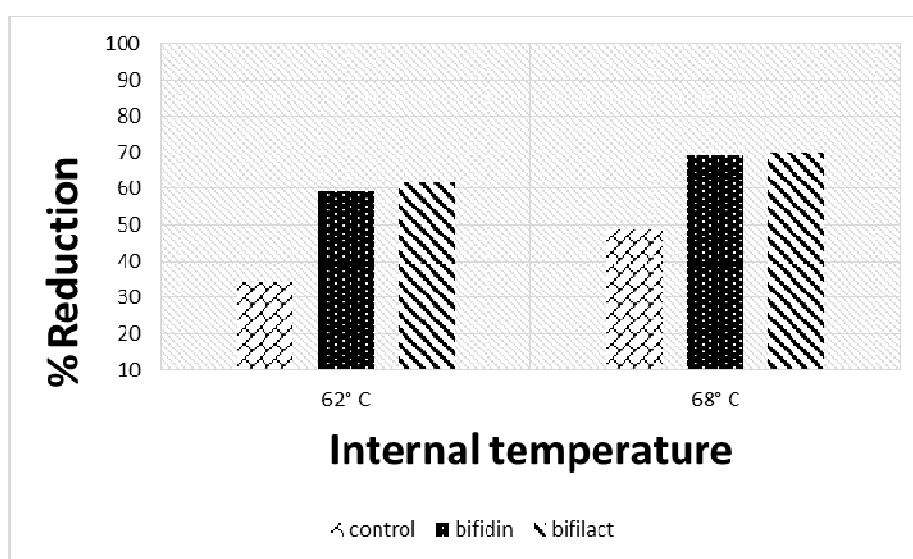


Fig. (11). Reduction ratio of *E. coli* 0157:H7 in ground beef patties (15% fat) treated with bacteriocin and grilled to an internal temperature of 62 or 68°C.

Table (4). Mean log CFU/g ($\pm$ standard deviation) of *E. coli* 0157:H7 in ground beef patties (8% fat), treated with bacteriocin and grilled to an internal temperature of 62 or 68°C.

internal temperature (°C)	Control		bifidin		bifilact	
	Count	% reduction	Count	% reduction	Count	% reduction
31	6.332 ^a $\pm$ 0.1	23.97	5.072 ^b $\pm$ 0.3	39.1	4.477 ^c $\pm$ 0.2	46.04
62	4.217 ^a $\pm$ 0.3	49.36	3.207 ^b $\pm$ 0.6	61.49	2.633 ^c $\pm$ 0.1	68.38
34	5.415 ^a $\pm$ 0.1	34.98	4.061 ^b $\pm$ 0.2	51.24	3.813 ^b $\pm$ 0.2	54.21
68	3.447 ^a $\pm$ 0.1	58.61	Zero ^b $\pm$ 0.0	100	Zero ^b $\pm$ 0.0	100

Mean in the same row with different superscripts are significantly difference at $p < 0.05$.

Initial number was 8.328 log CFU/g

Grill time at 62° C was 5.8 minutes

Grill time at 68° C was 7.4 minutes

Table (5). Mean log CFU/g ($\pm$ standard deviation) of *E. coli* 0157:H7 in ground beef patties (15% fat), treated with bacteriocin and grilled to an internal temperature of 62 or 68°C.

internal temperature (°C)	control		Bifidin		bifilact	
	Count	% reduction	Count	% reduction	Count	% reduction
31	7.041 ^a $\pm$ 0.1	15.18	5.462 ^b $\pm$ 0.3	34.21	5.301 ^c $\pm$ 0.2	36.14
62	5.447 ^a $\pm$ 0.2	34.38	3.382 ^b $\pm$ 0.2	59.26	3.176 ^b $\pm$ 0.2	61.74
34	6.342 ^a $\pm$ 0.5	23.61	4.362 ^b $\pm$ 0.3	47.45	4.322 ^b $\pm$ 0.5	47.93
68	4.248 ^a $\pm$ 0.3	48.83	2.580 ^b $\pm$ 0.3	68.92	2.477 ^b $\pm$ 0.3	70.16

Mean in the same raw with different superscripts are significantly difference at $p < 0.05$.

Initial number was 8.301 log CFU/g

Grill time at 62° C was 5.8 minutes

Grill time at 68° C was 7.4 minutes

Use of bifidin or bifilact Bb-12 (1.2 %) resulted in reduction of *E. coli* count by reduction ratio 100 % in sample containing 8 % fat when grilled to an internal temperature of 68°C, while these ratios decreased in sample containing 15 % fat (Fig. 10, 11). [20] reported that the chemical and physical properties of food, e.g. pH, and fat content, could also have a significant role in the suitability of a particular bacteriocin. The combination of lactic acid bacteria (LAB) was inhibitory to *L. monocytogenes* inoculated onto frankfurters not containing lactate/diacetate after 8 weeks of refrigerated storage (0.6 log reduction compared to *L. monocytogenes* only control), and when a cell free extract (CFS) of the LAB was added with LAB even more inhibition was obtained (1.2 log reduction compared with *L. monocytogenes* only). [67]

4. Conclusion

According to a large inhibitory spectrum and strong inhibitory activity, our results provide evidence that bifidobacteria exert antagonistic activity against pathogenic bacteria in vitro. In the present study, it was show that the inhibition of gram-negative pathogens bacteria was directly connected with the production of organic acids and other antibacterial compounds produced from bifidobacteria. The antimicrobial compounds can serve to increase food safety and prolong shelf life. The study also demonstrated that food service handling condition should then be optimized to place the organism in a more heat sensitive state, thereby maximizing the effectiveness of cooking treatments.

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