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Inhibition of *Bacillus cereus* in Fresh Guava-Nectar by Plantaricin and Nisin

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Abstract: In this study, the in *vitro* antimicrobial activities of some lactic acid bacterial strains (*Lactobacillus plantarum* ATCC 8014, *Lactococcus lactis* ssp. *Lactis* ATCC 11454) were investigated against an indicator strain *Bacillus cereus* DSM 351. Also, the stability of the inhibitory activity of the obtained bacteriocins was studied with different temperatures (30, 60, 90, 100 and 121°C), pH value levels (3-10) and the effect of proteolytic enzymes (pepsin, trypsin, protenase–K and α-amylase) using *B. cereus* as indicator strain. Applications of crude bacteriocins as alternative natural bio preservatives in guava nectar were determined to extend the shelf life of fresh guava nectar during storage at 5°C and at room temperature (25± 3°C) using *B. cereus* as indicator strain. Results showed that nisin was heat stable but plantaricin loss completely its activity above 30°C. Also, results indicated that nisin was stable at pH value ranging from 3 to 10 while plantaricin was inactive above 5 or below 4.Both nisin and Plantaricin were sensitive to proteinase enzyme, insensitive to α- amylase. While for trypsin, only the plantaricin was completely affected. plantaricin was sensitive to the pepsin where nisin was partially. Results also showed that nisin and plantaricin extended the shelf life of fresh guava nectar. Nisin was more effective at room temperature than plantaricin, while plantaricin was more effective at 5°C than nisin at room temperature. According to the Egyptian organization of standardization and quantity, E.s.1602-1/2005, results showed that plantaricin extend the shelf life at 5°C of guava nectar to the 8th day of storage, while nisin extend the shelf life to the 7th day of storage using a concentration of 1500 AU.ml-1 for both bacteriocins. At room temperature, plantaricin extend the shelf life of the nectar to 6th day of storage,while nisin extend the shelf life to the 9th day of storage using at the same concentration of 1500 AU.ml-1.Results indicated that plantaricin and nisin extended shelf life of inoculated guava nectar by *Bacillus cereus* to 8 and 6 days of storage at 5°C, respectively, while plantaricin and nisin helped in holding guava nectar within the limits of Egyptian organization of standardization and quality for 8 and 9 days at room temperature with the concentration at 1500 AU.ml^{-1}, respectively.

Key words: Lactic Acid Bacteria · Bacteriocins · *Bacillus cereus* · Guava Nectar

INTRODUCTION

In spite of modern advances in technology, reservation of foods is still a debated issue. Amelioration of economic losses due to food spoilage and satisfying the growing consumers demands for foods that are ready to eat, fresh-tasting, nutrient and vitamin and minimally-processed and preserved are major challenges for the current food industry [1].

Lactic acid bacteria (LAB) have an important role in preserving foods, preventing poisoning [2]. This inhibition is due to the production of various compounds as organic acids, diacetyl, hydrogen peroxide and bacteriocins [3].

Bacteriocins are generally recognized as safe (GRAS). Bacteriocins are a heterogeneous group of antibacterial proteins varying in the spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties [4].The interest in LAB bacteriocins due to the fact that they are produced by " food-grade" bacteria and might consequently be used as antimicrobial additives in food and feeds [5].

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Nisin, a typical bacteriocin of LAB produced by different *Lactococcus lactis*, has already been used in the food industry as antagonistic additive in more than 50 countries [6].

Bacillus cereus is an aerobic (facultative anaerobic) spore former bacterium that is widely distributed in the environment, mainly in soil from which it easily spreads to many types of foods, especially those of vegetable origin during harvesting, processing and handling [7].

This bacterium is one of the leading causes of food poisoning in the industrialized world, causing gastro intestinal disorders. Some strains of *B. cereus* are capable of producing a heat labile diarrheal enterotoxin and/or heat stable emetic enterotoxin and/or at least three different enterotoxins [8].

Therefore the objectives of this work were conducted to apply some produced bacteriocins by LAB in both inoculated and uninoculated fresh guava nectar of different concentrations, at 5°C and room temperature (25±3°C).

MATERIALS AND METHODS

Bacterial Strains: *Lactobacillus plantarum* ATCC 8014, *Lactococcus lactis* ssp. *Lactis* ATCC 11454 and *Bacillus cereus* DSM 351 were obtained from the Egyptian Microbial Culture Collection, (EMCC), Cairo Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University, Egypt.

Growth Conditions of Bacterial Strains: All strains used in this study were maintained as frozen stocks in 25% glycerol at -20°C. The cultures of producers, *L. plantarum,* were routinely propagated anaerobically in MRS broth at 37°C for 14-16 h, while *L.lactis* was cultivated at 32°C for 16-18 h in M17 broth according to De Man *et al.* [9]. The spoilage bacterial cultures were maintained at 4°C on agar slant and then they were sub cultured for a week using nutrient broth for all strains at 30°C and stored at 4°C until use.

Production of Crude Bacteriocins Samples: The cultures of LAB were subcultured anaerobically for 72 h, where *L. Plantarum* and *Lactococcus lactis* were cultivated in MRS broth at 37°C and M17 broth at 32°C, respectively.

Extraction of Bacteriocins: The cell free supernatants (CFS) containing the bacteriocins were obtained according to Abd El-basset and Djamila, [10] by centrifugation of cultures of LAB (7500 g for 10 min at 4°C), the CFS were adjusted to pH value 6.8 with NaOH 5M or HCl 5M to exclude the antimicrobial effect of organic acids, followed by sterilization of the cell free supernatant (CFS) through 0.45µm syringe filter cellulose acetate. Then**,** the inhibitory activity from H_2O_2 was eliminated by addition of 5mg/ml catalase, according to Daba *et al*. [11].

Determination of the Inhibitory Activity of Crude Cell Free Supernatant (CFS) on the Indicator Strain: The antimicrobial activity of the CFS was determined by the diffusion method of Tadesse *et al*. [12] as follows: Sterile filter discs (5mm) of Whatmann No.1 were inoculated with 50 µl of CFS and then placed on the solidified nutrient agar with an overnight culture of each indicator strain. According to Cabo *et al.* [13] the plates were kept at 4°C for 3-4 h to permit diffusion on the assay material then incubated at 30° C for 20-24 h. The results were recorded by measuring the diameter of the inhibition zones around the discs (in mm). The antibacterial activity tests were done in triplicates.

Determination of the Bacteriocins Titre: The culture supernatant was assayed for bacteriocin titre by spot on lawn technique as described by Barefoot and klaenhammer [14] with MRS agar using *Bacillus cereus* as indicator. The assay plates had pre-poured bottom layer of MRS agar (1.5%) and a top layer of MRS soft agar (0.7%) inoculated by diluted overnight culture (0.125ml of 10 fold diluted overnight culture to 5ml MRS soft agar 0.75%). Serial two-fold dilutions were carried out in the medium used for the growth of the indicator strain. Wells in the test plates were inoculated by 50 µl of each dilution. The plates were incubated at 30°C for 24 h and examined for the presence of 2mm or larger clear zones of inhibition around the wells.

According to Graciela *et al*. [15] the antimicrobial activity of bacteriocin (titre) is defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn; and expressed in arbitrary units (AU. ml^{-1}). For each experiment three replicates were used.

Characterization of Inhibitory Substances in the Supernatant: Cell free supernatants (CFS) from lactic acid bacteria were adjusted at pH value 6.5 and exposed to heat treatments of 30 and 60°C for 30, 90 and 100 min and 121°C for 20 min. The remaining activities were determined by well diffusion assay using *B. cereus* as indicator strain.

In order to determine the effect of pH value, the CFS containing the bacteriocins were adjusted to pH values in the range of 3 to 10. The pH value adjusted bacteriocin samples were incubated at 37°C for 20 min, then neutralized to pH value 7 and tested for bacteriocin activity. The following enzymes were tested for their hydrolytic activity on the antimicrobial compounds contained in the supernatants (pepsin –proteinase -K, trypsin and α -amylase). The assays were performed at final concentration (1 mg /ml) and at pH value 6.8 except pepsin at pH value 3.0 as described by Abd El-basset and Djamila [10]. Samples of bacteriocins (0.5 ml) incubated with (0.5 ml) of each enzyme and without enzymes were held at 37°C for 2 h, then testing the remaining activity using well diffusion assay (WDA) against *B. cereus*.

Preparation of Fresh Guava Nectar: Guava fruits were washed, cut into halves and water was added and blended with mixer (Osterizer, Oster ® Brand, USA) to obtain a homogenized guava puree. Citric acid was added at 0.14% during homogenizing, then filtrated with fine mesh nylon cloth and pressed by hand to remove the seeds from guava puree. Guava nectar was prepared from guava puree by diluting with water and adding sucrose to adjust total soluble solids to give a (T.S.S.) 14% of the final product having 50% guava juice.

Application of Bacteriocins: The tested bacteriocins were applied on both uninoculated and inoculated fresh guava nectar with Bacillus cereus using (MYP agar) as selective media at 5°C and room temperature 25±3°C.

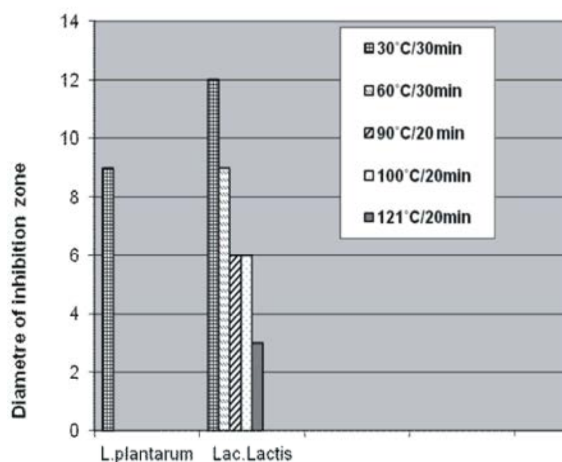

Fig. 1: Thermal sensitivity of bacteriocins against *Bacillus cereus* using well diffusion assay.

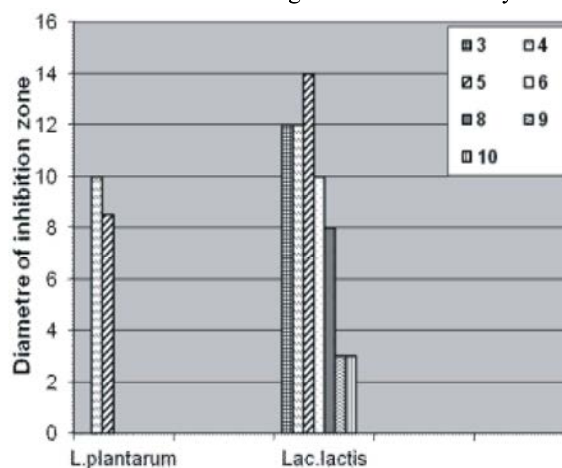

Fig. 2: pH-changeseffect on bacteriocins against *Bacillus cereus* well diffusion assay.

RESULLTS AND DISCUSSIONS

Characterization of Bacteriocins: With regard to thermal sensitivity, data present in Fig. 1 show that plantaricin lost activity above 30°C. These findings indicated that the inhibitory compound was heat labile as recorded by several investigators. Nisin was heat stable at 100°C/20 min, whereas a great reduction in activity was recorded at 121° C/20 min. These results are in accordance with Hurst [16] who stated that nisin loses 90% of its activity at 115°C with pH value 6.8. In addition, Oscariz and Pisabarro [17] explained the heat stability as a major feature of low molecular weight bacteriocins due to the presence of disulphide intramolecular bonds which stabilize secondary structures by reducing the number of possible unfolded structures. Also, results indicated that plantaricin was instable at pH value above 5 or below 4, but nisin was stable at pH value ranging from 3 to 10 in Fig. 2. These results might be attributed to the isoelectric point of these bacteriocins which is directly affected by the pH value of the media and it affects by its turn the antimicrobial activity of the bacteriocins, as explained by Mortvert-Abildgaard *et al*. [18]. Results in Fig. 3 should that both bacteriocins were Sensitive to proteolytic enzymes and insensitive to α-amylase. While, nisin was partially affected by pepsin, insensitive to trypsin, these results are in agreement with those of Tatsadjeu *et al*., [19].

The ability of foodborne pathogens to contaminate fruits and vegetables has led to impose hazard analysis and critical control point requirements on juice processors [20].

Table 1: Effect of different concentrations of bacteriocins during storage at 5°C on total viable count (logCFUml^{-1}) of fresh guava nectar.

Storage (Days)	Conc. Plantaricin concentrations 0 AU.ml^{-1}	500AU.ml^{-1}	1000AU.ml^{-}	1500AU.ml^{-1}	Nisin concentrations 0 AU.ml^{-1}	500 AU.ml^{-1}	1000 AU.ml^{-1}	1500 AU.ml^{-1}
0	2.07	2.24	2.09	2.14	2.07	2.20	2.07	2.09
1	2.00	1.39	-	-	2.00	-	-	-
2	1.90	-	-	-	1.90	-	-	-
3	2.30	-	-	-	2.30	-	-	-
4	2.37	1.37	-	-	3.37	1.32	1.10	-
5	3.34	1.50	1.39	-	3.34	2.24	1.97	-
6	3.66	1.63	1.90	-	3.66	2.81	2.50	1.67
7	3.66	2.19	1.90	1.36	3.66	2.92	2.73	1.72
8	3.81	2.21	2.14	1.97	3.81	2.90	2.97	2.07
9	3.83	3.19	2.34	2.41	3.83	3.37	2.99	2.32
10	3.84	3.30	2.43	2.44	3.84	3.52	3.05	2.55
11	4.00	3.65	2.49	2.50	4.00	3.86	3.10	2.83
12	4.22	4.07	2.51	2.49	4.22	4.20	3.19	3.02

1AU: One arbitrary unit Count: logCFU.ml^{-1} complete inhibition

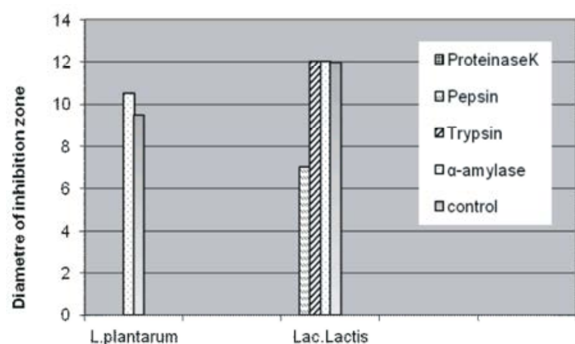

Fig. 3: Sensitivity of bacteriocins to enzymes activities against *Bacillus cereus* using well diffusion assay.

Effect of Plantaricin and Nisin Concentrations on the Total Viable Counts of Fresh Guava Nectar at 5°C: Results in Table 1 indicated that addition of 500 AU.ml^{-1} of plantaricin showed an obvious gradual decrease in viability extent to the 6th day. Increasing the concentration of plantaricin to 1000 and 1500AU. ml^{-1} totally inhibited the growth for four up to six days of storage, respectively, followed by a few viable cells (with 1500AU. ml^{-1}), which might be originated from a very low number of survivors. Thus, this last concentration seemed to be the most effective in extending the shelf life of nectar to the 8th day.

The potential of microbial inhibition of nisin was obvious by adding 500 AU.ml^{-1}, where no surviving population was detected for the first three days. Whiles, the number of cells recovered reappeared in increasing manner till the end of the storage period. The highest inhibition period was obtained by adding 1500 AU.ml^{-1} for 5 days with no viable cells detected. Moreover, to the 7th day the surviving population was markedly low ranging from 1.67 to 1.72 log CFU.ml^{-1}. These results are in agreement with the Egyptian organization of standardization and quality [21] for the non –carbonated sweeten drinks for fruit nectars who stated that the total viable count should not exceed 100 cells /ml.

The afore-mentioned results confirmed the effectiveness of nisin as preservative in fruit nectar. A recent study by Pathanibul *et al*. [22] who demonstrated the effectiveness of nisin as non thermal technology for the inactivation of microorganisms as *Escherichia coli* and *listeria innocua*, in fruit and vegetable juices and the synergism of nisin with the high pressure homogenization in apple and carrot juices. The primary action site of nisin against vegetative cells is considered to be the cytoplasmic membrane, where nisin acting as voltage dependent polarizer [23]. In spite of the clear effectiveness of nisin from the obtained results with small concentration in the present work, its inhibitory capability was lost by increasing the storage time. These findings are consistent with the work of several other investigators who studied nisin antimicrobial activity. It has been reported that nisin loses its inhibitory activity with time [6].

Effect of Plantaricin and Nisin Concentrations on Total Viable Counts of Fresh Guava Nectar at Room Temperature (25±3°C): Results from Table 2 show the general decrease in plantaricin potential by increasing the temperature near the maximal limits for its function, however the increase in concentration from 500 to 1500 AU. ml^{-1} could increase its effectiveness and extend the

Table 2: Effect of different concentrations of bacteriocins during storage at room temperature on total viable count (logCFU.ml^{-1}) of fresh guava nectar

Storage	Conc.							
	Plantaricin concentrations				Nisin concentrations			
(Days)	0 AU.ml^{-1}	500AU.ml^{-1}	1000AU.ml^{-}	1500AU.ml^{-1}	0 AU.ml^{-1}	500 AU.ml^{-1}	1000 AU.ml^{-1}	1500 AU.ml^{-1}
0	2.07	2.24	2.22	2.20	2.07	2.24	2.24	2.17
1	2.22	1.24	-	-	2.22	-	-	-
2	2.91	1.52	-	-	2.91	-	-	-
3	3.11	1.99	-	-	3.11	-	-	-
4	3..45	2.31	1.98	1.07	3.45	-	-	-
5	3.62	2.67	2.25	1.40	3.62	1.94	-	-
6	3.98	2.77	2.53	1.87	3.98	1.94	1.30	-
7	4.37	2.93	2.68	2.34	4.37	2.02	1.60	1.63
8	4.62	3.10	2.78	2.65	4.62	2.20	1.94	1.30
9	4.88	3.34	3.05	2.73	4.88	2.53	2.32	1.67
10	5.12	3.71	3.42	2.84	5.12	2.69	2.55	2.25
11	5.34	3.90	3.73	3.17	5.34	2.98	2.79	2.63
12	5.66	4.39	4.04	3.41	5.66	3.27	3.16	2.71

1AU: One arbitrary unit. Count: logCFU.ml $^{-1}$ Complete inhibition

shelf life of the nectar. Where, at room temperature a relatively stable reduction for the first three days indicating the inhibitory effect of this bacteriocin. This reduction was followed by a rapid and linear increase of surviving population which was favoured by the room temperature suggesting the deficiency of this bacteriocin to preserve and to extend the shelf life of the nectar over six days even at 1500AU.ml-1 at this temperature. Previous research on bacteriocins effectiveness, reported that viable cell counts decrease gradually over incubation time, depending upon the bacteriocin concentration, the temperature of incubation and the food sample [24].

Bacteriocin activity and stability in foods may be influenced by several factors as reported by Ganzle *et al.* [1] including the pH value of food, the food composition and the temperature of incubation.

For nisin the most striking was the magnitude of reduction which was more pronounced than at 5°C suggesting that nisin activity increased as the temperature increased.

Once again, the lethal effect of nisin declined gradually with increasing time where the number of recovered cells increased in the nectar, exceeding the initial population during subsequent storage reaching 3.27, 3.16 and 3.02 log CFU.ml^{-1} with 500, 1000 and 1500 AU.ml^{-1}, respectively. These results are in harmony with those demonstrated by Jung *et al*. [25] and Abee *et al*. [26] who showed that the action of nisin Z (1 µg / ml) against *Listeria monocytogenes* cells is severely reduced at decreased temperatures and similar findings have been reported for the same microorganism [27].

The obtained data were explained by Abee *et al*. [26] indicating that at low temperature there are changes in the membrane that affected the target of nisin enabling the access to these sites. The ordering of the lipid hydrocarbon chains, which occur at low temperatures results in a decrease of membrane fluidity which are probably responsible for the reduced nisin Z efficiency observed on *L. monocytogenes*. On the other hand, alternations in cytoplasmic membrane properties or alternations in the peptidoglycan may modulate nisin sensitivity.

Effect of Plantaricin and Nisin Concentrations on the Total Viable Counts of Inoculated Guava Nectar by *B. Cereus* at 5°C: It could be noticed in Table 3 that in inoculated fresh guava nectar not supplemented with bacteriocins at 5°C, the initial number of recovered cells undergo a great reduction which reached 2.50 log CFU.ml^{-1} at the first three days indicating a low tolerance to the decrease in temperature since 5°C is below the temperature limits for *B. cereus* growth but after a period of adaptation the viable counts increased gradually. By adding 500 AU.ml^{-1} of plantaricin, the size of surviving population decreased slowly from 5.84 to 1.63 log CFU. ml^{-1} then restarted the proliferation. The maximum yield of complete inhibition detected from the 1st day of application till the 7th day of storage was obtained by adding 1500 AU. ml^{-1} to the nectar and it remained of low viable count and safe for subsequent 8th day. Abriouel *et al*. [28] stated that the greater efficacy of bacteriocin enterocin AS-48 against *B. cereus* at 5°C could be attributed to a slowing of growth caused by the low

Table 3: Effect of different concentrations of bacteriocins during storage at 5°C on total viable count (logCFU.ml^{-1}) of fresh guava nectar inoculated by *Bacillus cereus*

	Conc.							
	Plantaricin concentrations				Nisin concentrations			
Storage Per.(days)	0 AU.ml^{-1}	500AU.ml^{-1}	1000AU.ml^{-1}	1500AU.ml^{-1}	0 AU.ml^{-1}	500 AU.ml^{-1}	1000 AU.ml^{-1}	1500 AU.ml^{-1}
0	5.89	5.84	5.90	5.89	5.89	5.89	5.69	5.69
1	3.40	1.82	-	-	3.40	2.01	-	-
2	3.36	-	-	-	3.36	-	-	-
3	3.30	-	-	-	3.30	-	-	-
4	3.77	-	-	-	3.77	1.62	-	-
5	3.81	1.63	-	-	3.81	1.96	1.93	-
6	4.25	2.06	1.20	-	4.25	2.15	1.97	1.71
7	4.49	2.31	1.79	-	4.49	2.49	2.40	2.00
8	4.80	2.56	2.22	1.33	4.80	2.89	2.76	2.62
9	5.31	2.84	2.57	2.03	5.31	3.40	3.34	2.92
10	5.77	3.42	2.82	2.45	5.77	3.93	3.66	3.51
11	5.82	3.86	3.17	2.73	5.82	4.34	3.94	4.01
12	6.01	4.03	3.32	3.03	6.01	4.52	4.25	4.10

1AU: one arbitrary unit Count : (logCFU.ml $^{-1}$) Complete inhibition

Table 4: Effect of different concentration of bacteriocins during storage at room temperature on total viable count (logCFU.ml^{-1}) of fresh guava nectar inoculated by *Bacillus cereus*

	Conc.							
	Plantaricin concentrations				Nisin concentrations			
Storage (Days)	0 AU.ml^{-1}	500AU.ml^{-1}	1000AU.ml^{-}	1500AU.ml^{-1}	0 AU.ml^{-1}	500 AU.ml^{-1}	1000 AU.ml^{-1}	1500 AU.ml^{-1}
0	5.65	5.60	5.82	5.82	5.65	5.80	5.80	5.69
1	5.69	3.00	1.83	-	5.69	0.87	-	-
2	5.84	-	-	-	5.84	-	-	-
3	5.77	-	-	-	5.77	-	-	-
4	5.85	1.66	-	-	5.85	-	-	-
5	6.01	2.03	1.77	1.12	6.01	-	-	-
6	6.00	2.27	2.14	1.88	6.00	1.83	1.66	-
7	6.07	3.01	2.54	2.30	6.07	1.84	1.72	0.97
8	6.20	3.53	2.96	2.67	6.20	2.23	1.96	1.66
9	6.17	3.96	3.38	2.92	6.17	2.70	2.33	1.98
10	6.17	4.32	3.80	3.44	6.17	3.13	2.73	2.24
11	6.33	4.97	4.25	3.90	6.33	3.62	3.14	2.51
12	6.52	5.44	4.67	4.22	6.52	4.04	3.27	2.98

1AU: One arbitrary unit Count: (logCFU.ml^{-1}) Complete inhibition

temperature, which resulted in a higher number of bacteriocin molecules per cell as well as longer exposure of sublethally injured cells to the damaging bacteriocin.

Applying increasing concentration of nisin, at 5°C, resulted in a reduction of the viable count of cells, which was rapid to achieve complete inhibition within 24h. These complete reductions in viable count extended to the 3rd, 4th and 5th day for 500, 1000 and 1500 AU.ml^{-1}, respectively, these reductions became less evident when storage time was increased and nisin began losing its activity, thus the total viable counts exceeded the safety limits of the Egyptian organization of standardization and quality [21], hampering the product to meet the economically important shelf –life.

Comparing the reductions in viable counts in Table 2 of non inoculated nectar treated by nisin at the same temperature, it is obvious that reductions were relatively less pronounced in table 3 suggesting the presence of factors that might affect the nisin activity which may be attributed to the larger innocula or the enzymatic activity of the target organism. Where many recent researches reported that several factors may affect nisin, thus lessening its availability for antibacterial activity. Nisin is

more active aginst *B. cereus* as pH value decreases, but it may be degraded by proteolytic enzymes originating from plant or animal tissues or from microorganisms [29].

Delves *et al*. [6], Javris [30] and Javris and Farr, [31] reported that nisin loses its inhibitory activity with time and that several Bacillus species produce a dehydropeptide reductase which breaks down nisin.

Effect of Plantaricin and Nisin Concentrations on the Total Viable Counts of Inoculated Fresh Guava Nectar at Room Temperature (25±3°C): Results in Table 4 show the effect of adding plantaricin and nisin at different concentrations. Once more, a great loss in activity of the plantaricin occurred at this temperature compared to its reductions at 5°C. Results in tables 3 and 4 showed that reduction of viable counts were followed by an exponential increase for the subsequent days reaching higher viable counts at room temperature than at 5°C, this fast outgrowth of the pathogen *B.cereus* was favoured by the optimal temperature for growth. Grande *et al.* [24] had applied enterocin AS-48 in several fruit and vegetable juices. They had stated that interaction between AS–48 and vegetable food components must be studied before application. Enterocin AS- 48 is very stable in some vegetable juices for the first 24 to 48 h of storage under refrigeration and decay of activity is markedly influenced by the storage temperature. While in fruit juices and juice mixtures AS-48 are stable for 15 days at 4°C. Gradual and variable loss of activity occurs in juices stored at 15 and 28°C; inactivation was faster at higher temperature. It could be noticed from the magnitude of reductions that nisin effectiveness was greater at room temperature than at 5°C.

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