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Synergistic Effect of Nisin and Cinnamaldehyde against Alicyclobacillus Acidoterrestris in Orange Nectar.

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ABSTRACT

The present study was undertaken to evaluate the effect of nisin and cinnamaldehyde alone and in combination on the D-values of *Alicyclobacillus acidoterrestris* spores using different temperatures. The effect of sublethal concentration of antimicrobials, on D values were selected to be applied in both pasteurized and unpasteurized orange nectar stored at temperature (25-45 °C). Cinnamaldehyde effectiveness was dose dependent showing the sharper decrease in D-values (7-1.7) min at (90-95°C) respectively using 0.5 µl/ml of cinnamaldehyde .While, nisin displayed a reduction in D-values (9.2-3.6) min at (90 – 95° C), respectively using 62.5 IU/ml above which no significant decrease was recorded. Their combination revealed the most pronounced decrease in the D-values (7.65-3.8) min at (90 – 95° C) using 0. 26 µl/ml and 31.25 IU/ml of cinnamaldehyde and nisin, respectively. Where the combination (46.8 $IU.ml^{-1}$/0.39 $\mu l.ml^{-1}$) of nisin/cinnamaldehyde was potential in extending the shelf of pasteurized orange nectar stored at 45° C to the 33 th day compared to the unpasteurized nectar to the 21 th day at the same temperature of storage. While, the synergistic effect of this combination help in extending the shelf life , at 25° C of storage to 45 th day in pasteurized orange nectar compared to the unpasteurized nectar to 27 th day of storage.

Keywords: *Alicyclobacillus acidoterrestris*, Synergistic ,orange nectar ,nisin, cinnamaldehyde.

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INTRODUCTION

Alicyclobacillus acidoterrestris is considered to be one of the important target microorganisms in the quality control of acidic canned foods. There is an urgent need to develop a suitable method for inhibiting or controlling the germination and outgrowth of *A .acidoterrestris* in acidic drinks [1].

Generally, fruit products, such as juices, nectars, concentrates or purees, are acidic (pH, 4.6) and therefore, a pasteurization treatment in the temperature range of 85-95°C should be adequate for their stabilization at ambient temperature [2]. Another process should be added to the thermal treatment, since high temperature may cause the loss of the product's sensorial and nutritional characteristics [3]. The food industry is attempting to move away from the use of chemical preservatives .New approaches, like the use of natural compounds or non-thermal methods [4]. While maintaining microbiological safety currently, there is a strong debate about the safety aspects of chemical preservatives since they are considered responsible for much carcinogenic as well as residual toxicity. For these reasons, consumers tend to be suspicious of chemical additives and thus the demand for natural and socially more acceptable preservatives has been intensified [5]. Nowadays, Western countries are experiencing a trend of green consumerism, desiring fewer synthetic additives and mores friendly compounds. Essential oils have been long recognized for their antibacterial, antifungal, antiviral, properties [6].

However, there are some limitations for the use of essential oils or nisin. Where, higher concentrations of essential oils are required in foods than in laboratory media; accordingly, the practical application of essential oils is restricted, since the addition of high concentrations of the oils causes' undesirable flavor changes in the food product [7]. Nisin is an expensive substance and hence the cost of commercial use of this agent may be prohibitive for many industries. Also, the use of high concentrations of nisin may encourage the selective growth of nisin-resistant bacterial sub-population [8].These problems could be overcome by using other agents in combination with nisin resulting in synergistic effects, thus requiring low concentrations of nisin for effective control of the relevant microorganisms [9]

Therefore, the present study was aimed to evaluate the effects of cinnamaldehyde and nisin and their combination on the reduction time (D-value) of the *A .acidoterrrestris* spores .Moreover, the effect of addition of nisin and cinnamaldehyde alone or combined as a hurdle technology was assessed on shelf life of orange nectar.

MATERIALS AND METHODS

Materials

Nisin (Nisaplin) was purchased from Sigma –Aldrich Chemical Co.(N5764;Sigma, St. Louis ,Missouri ,USA) that contains 2.5% nisin with minimum potency of 10^6 U/g**.** Cinnamaldehyde was purchased from Sigma-Aldrich chemicals. The purity of the component was the highest available (93 %) with code 101196701and density 1.05g/ml at 25° C**.** *Alyclobacillus acidoterrestris* was obtained from The American Type Culture Collection (ATCC) 49025.Valencia orange (*Citrus sinensis*) was purchased from local supermarket, Giza, Egypt.

Methods

Bacterial strain and culture medium

The bacterial strain was grown and maintained on malt extract agar (MEA) slants acidified to pH 4.5 through a sterile solution (1:1, w /w) of citric acid , containing: malt extract(17.5g/l), peptone (3g) [10].

Preparation of spore suspension

Spore crops were obtained according to Beuchat *et al* [11] and Sinigaglia *et al* [12] as follows: Cultures grown in MEA broth for 48h were surface spread on a solid sporulation medium consisting of malt extract agar (MEA) supplemented with 0.05 g/l of $MnSO_4$ and incubated for 5-7 days at 45°C to obtain at least 90– 95% sporulated, the level of sporulation was monitored by staining the spores with a solution of 5% malachite green and then with 0.5% safranin followed by examination under light microscopy. Spores were

harvested by flooding the agar plat surface with sterile distilled water then gently scraping off with sterile bent glass rod and resuspended in sterile distilled water (3ml per plate). The pool of spores collected from the different plates was centrifuged at 5000g for 15 min at 4°C, washed two times with sterile distilled water by repeated centrifugation , and finally resuspended in sterile distilled water (6– 7 log units/ ml, as determined by plating on MEA) and stored in Eppendorf tubes at 22°C until use as intact, non- activated spore suspensions . This suspension was activated to germinate by heating at 80 °C for 10 min followed by 1h incubation on ice. The number of spores was determined by serially diluting the heat-shocked spore suspensions and the content was plated in triplicate on MEA . Plates were incubated for 5 days at 45 °C and the grown colonies were counted with an initial bacterial load of 2.10^8 sp/ml.

Preparation of orange nectar

The oranges were carefully washed with a sponge and neutral detergent, then rinsed and immersed in a hypochlorite solution (100 mg/L of free chlorine) for 15 min. They were then transported to a laminar flow chamber and rinsed in sterile distilled water. After wiping with sterile towels, the fruits were cut with disinfected knives and the juice extracted in a domestic juicer. The orange nectar was prepared from orange juice by diluting water and adding sucrose to adjust T.S.S give a final product having 50% range juice where total soluble solids was 16%. The nectar was stored and frozen at 18 °C in sterile containers. The nectar was pasteurized under aseptic conditions (90 °C for 15 s) and filled into paperboard packaging without the addition of chemical preservatives. Before evaluating the incidence of Alicyclobacillus spp in the juices, and then antimicrobials were added.

Determination of D- values

Decimal reduction time (D_t) values were determined according to Pena *et al* [13]and Bevilacqua *et al* [14]. Thermal processing was applied under three different temperatures (85, 90 and 95 °C) for each sample for each antimicrobial as shown in Table (1). Different concentrations of each antimicrobial were tested as a criterion in order to determine the maximum concentration that minimizes the D- values for the spores. Stock solutions of cinnamaldehyde and nisin (0.26 ; 0.52 ; 1.04; 2.09 and 4.18µl.ml^{-1}),(15.62 ; 31.25; 62.5 ; 125and 250IU.ml^{-1}); respectively, were freshly prepared . The experiment was carried out on activated spores at (80 °C for 10 min) than the spore suspension was leafed in the ice bath for 1h.The number of viable spores was determined by serially diluting in sterile saline solution than spreaded by triplicate on MEA. Plates were incubated for 45°C for 4 days, and the grown colonies were counted.

Table1.Factorial planning for thermal inactivation for *A.acidoterrestris*

Variables					
Experiment	**T[a](°C)**	**Ni[b] IU/ml**	**Experiment**	**T[a] (°C)**	**CNMA[c]**
1	85	0	**19**	85	0
2	85	15.62	**20**	85	0.26
3	85	31.25	**21**	85	0.52
4	85	62.5	**22**	85	1.04
5	85	125	**23**	85	2.09
6	85	250	**24**	85	4.18
7	90	0	**25**	90	0
8	90	15.62	**26**	90	0.26
9	90	31.25	**27**	90	0.52
10	90	62.5	**28**	90	1.04
11	90	125	**29**	90	2.09
12	90	250	**30**	90	4.18
13	95	0	**31**	95	0
14	95	15.62	**32**	95	0.26
15	95	31.25	**33**	95	0.52
16	95	62.5	**34**	95	1.04
17	95	125	**35**	95	2.09
18	95	250	**36**	95	4.18

[a]Temperature; [b] Nisin; [c] cinnamaldehyde

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Preparation of nisin and cinnamaldehyde combination in malt extract agar.

After determining the maximum antimicrobial concentration for each antimicrobial giving the best decrease in D-value, the sublethal concentration of nisin was selected and combined with the least effective concentration of cinnamaldehyde. The effect of this combination was tested again on the D values of *A. acidoterrestris* spores.

Nisin and cinnamaldehyde combination in orange nectar.

The last combination of nisin and cinnamaldehyde applied in laboratory medium Malt extract agar designed as(MEA) and its multiple concentrations (1.5MEAand 2MEA)were then applied in orange nectar.

Synergistic effect of nisin and cinnamaldehyde to extend shelf-life of orange nectar

The most effective combination from (MEA, 1.5MEA; 2MEA) of nisin and cinnamaldehyde and their individual concentrations were selected to be performed in both pasteurized and unpasteurized orange nectar and stored at 25-45° C.

Statistical analysis.

The data was statistically analyzed using ASSISTAT Version 7.7 beta software using subdivided parcels experiment with two factors (concentration and temperature .Tukey test at 5% level of probability was used to compare means of treatments [15].

Preparation of stocks solutions of nisin

Nisin stock solution was prepared by dissolving (100mg in 100ml 0.02N HCl) to give the concentration of 10^3 IU/ml. Then the solution was sterilized by autoclaving at 121°C for 15min, and stored at -20° C until used .Prior to experiments, the stock solution was thawed at 25° C. Two serial fold dilution were obtained in sterile water from1000 to reach 15.62 IU/ml.

Preparation of cinnamaldehyde

Stock solution of cinnamaldehyde (0.25Mole/33.5µl.ml^{-1}) were prepared in ethanol/water solution (1:1V/V), filtered, the pore of the membrane was 0.22 µm) and used immediately **.**This stock solution was diluted two serial fold dilution to reach 0.13 µl.ml^{-1}from which tested concentration were used.

RESULTS AND DISCUSSION

Effect of nisin on D values of *A. acidoterrestris* spores.

Alicyclobacillus spores may survive the pasteurization treatment [13].Thus results in **Table (2)** summarize the effect of nisin concentrations at different temperatures on the heat resistance of *Alicyclobacillus acidoterrestris* spores. It could be noticed that with the addition of 15.62 IU/ml (C_1), the D value decreased by 8.1% of the resistance obtained in the control (0 IU/ml), and as the nisin concentration increased the decrease in the D values became apparent this may be explained by Valero and Frances [16] who indicated that micro-organisms are sometimes unable to overcome the combination of several hurdles like mild heat treatments, acidic pH values and the addition of antimicrobials from natural origin, these ideas could result in a stable and safe product without any loss of sensorial quality.

The more evident at concentrations up to 62.5 IU (with a reduction of 28.6% in the D value), above this value, there was no significant difference of decrease in heat resistance compared to 125 IU/ml, indicating that the increase in the concentration did not improve the ratio of reduction.

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Table 2: Effect of nisin on the D-value of *Alicyclobacillus acidoterrestris* spores in malt extract agar.

Heat treatment	85 ºC				90 ºC				95 ºC			
Concentration ($IU.ml^{-1}$)	D(min)[a]	±SD[b]	R^2	%Reduction	D(min)	±SD	R^2	%Reduction	D(min)	±SD	R^2	%Reduction
C_0 (0)	38.4 ^{aA}	0.55	0.71	———	15.3 $^{a\,B}$	0.9	0.95	———	6.25 ^{aC}	0.47	0.96	———
C_1 (15.62)	34.9 ^{bA}	2.7	0.71	8.1%	13.8 ^{abB}	1.6	0.95	9.8%	5.9 ^{aC}	1.08	0.89	4.8%
C_2 (31.25)	28.2 ^{cA}	0.98	0.93	25%	10.3 ^{bcB}	0.98	0.97	32.6%	4.1 ^{aC}	1.25	0.92	33.8%
C_3 (62.5)	25.1 ^{cA}	1.27	0.85	28.6%	9.2 ^{cB}	1.15	0.96	39.8%	3.6 ^{aC}	1.09	0.93	40.3%
C_4 (125)	24 ^{cA}	1.1	0.82	36.8%	9.2 ^{cB}	2.2	0.96	39.8%	3.3 ^{aC}	1.35	0.92	47.2%
C_5 (250)	24.3 ^{dA}	2.5	0.71	36.0%	9.0 ^{cB}	1.05	0.91	41.1%	3.4 ^{aC}	0.86	0.94	45.6%

a)D:Decimal reduction time ; b)Values are means± standard deviations .With each column, means with the same small letter are not significantly different and each row, means with the same capital letter are not significantly different ($p > 0.05$).

Table 3: Cinnamaldehyde effect on the D-value of *Alicyclobacillus acidoterrestris* spores in malt extract agar.

Heat treatment / Concentration µL.ml^{-1}	85 ºC				90 ºC				95 ºC			
	D(min)[a]	±SD[b]	R^2	%Reduction	D(min	±SD	R^2	%Reduction	D(min)	±SD	R^2	%Reduction
C_0 (0)	38 $^{a\,A}$	0.55	0.71	———	15.2 $^{a\,B}$	0.9	0.95	———	6.25 $^{a\,C}$	0.47	0.96	———
C_1 (0.26)	31.8 ^{bA}	1.86	0.72	16.3%	13.6 $^{a\,b\,B}$	1.11	0.97	10.5%	5.2 ^{abC}	0.98	0.93	16.1%
C_2 (0.52)	23.2 $^{c\,A}$	1.0.	0.89	38.9%	11.7 $^{b\,B}$	1.5	0.98	23.0%	4.1 $^{a\,b\,c\,C}$	0.98	0.90	33.8%
C_3 (1.04)	18.5 $^{d\,A}$	2.35	0.83	51.8%	8.2 $^{c\,B}$	1.21	0.89	46%	2.6 ^{bcC}	1.5	0.82	58.0%
C_4 (2.09)	17.2 $^{d\,A}$	1.11	0.82	54.7%	8 $^{c\,B}$	1.9	0.93	47.3%	2.1b $^{c\,C}$	1.47	0.91	66.4%
C_5 (4.18)	16 $^{d\,A}$	1.47	0.89	57.9%	7 ^{cB}	1.6	0.98	53.9%	1.7 ^{cC}	1.11	0.93	72.8%

a)D:Decimal reduction time ; b)Values are means± standard deviations with each column, means with the same small letter are not significantly different and each row, means with the same capital letter are not significantly different ($p > 0.05$)

From the represented data in Table (2) it could be noticed that the reduction percentages becomes more evident as the tested temperature becomes higher ,where the maximum reduction attained 47.2 % at 95º C at 125IU/ml . Nevertheless, to the same nisin concentration at lower temperature, it is to be noted that , the reduction was not significant compared with the lower concentrations suggesting that this recorded reduction in percentage at 95º C was not attributed to the elevation of nisin concentration but is the result of the higher temperature . These results are in accordance with the finding of [17] who stated that the more the spores are heat damaged the more they are susceptible to nisin, where nisin appears to bind to sulphydryl groups on the spore surface. The nisin action against spores was sporicidal rather than sporostatic, and nisin seemed to inhibit the germination process at the stage of preemergent swelling [18].

Effect of cinnamaldehyde on D values of *A. acidoterrestris* spores.

Results in **Table (3**) show that there was a fast decrease in resistance with the addition of (C_1) 0.26 µl/ml , which only represents 16.3% of the resistance obtained in the control (0 IU/ml), and as the concentration increased the decrease in the D value became more apparent. The effect was more pronounced at concentration (C_3) 1.04 µl/ml where the higher amount of the active compound was used as evidenced by a sharper increase in the percentage of reduction (51.8%, up to C_5 (0.5) to a reduction of 72.8% at 95°C indicating that its effectiveness was concentration dependent. The use of essential oil (Eo_s) to control the germination of *A. acidoterrestris* spores was proposed by [19].

As demonstrated by Feron *et al* [20] that in cinnamaldehyde an aldehyde groups are reactive and have the ability to cross-link covalently with DNA and proteins through amine groups, thereby interfering with their normal function. The possibility of enhancing the antimicrobial activity of cinnamaldehyde by combining different temperatures, in order to point out a low dose with an effective inhibition of spores germination at mild temperature. Some reports have stated to support our findings.

Where, Bevilacqua *et al*[10] stated that the heat shock resulted in a slight decrease of *Alicyclobacillus acidoterretris* spore number and occurred at 80–85 °C depending on the pH of the medium. Otherwise, cinnamaldehyde acted as an additional hurdle within the storage time. In addition, Bevilacqua *et al* [21] reported that the inhibition of *Alicyclobacillus acidoterrestris* (c8 and c24) spores through the use of cinnamaldehyde was dose-dependent.

Effect of combinations between cinnamaldehyde and nisin on the heat resistance of activated spores in malt extract agar

Results in (Table 4) indicate that at the combination of cinnamaldeyde and nisin revealed a significant decrease in D values (7.65± 0.66 min) showing a reduction percentage of 50% with the thermal treatment at 90°C, comparing to 32.6% and 10% for the same concentration at the same temperature for nisin and cinnamaldehyde individually, respectively demonstrating that the sub- inhibitory concentrations used exhibited synergism against *A.acidoterretris*. This reduction increase to 3.8 min as the temperature increase to 95°C. Thus showing synergistic effect and the antimicrobial activity of nisin was greatly potentiated by the cinnamaldehyde.

The same trend of synergitism was shown by Ettayebi *et al* (8) who stated that the concentration of nisin required for effective control of food-borne pathogenic bacteria could be considerably lowered by the use of thymol in combination thus nisin effect was greatly potentiated by sub-inhibitory concentrations of thymol.

Effect of Cinnamaldehyde and nisin combinations in orange nectar.

The last combination of nisin and cinnamaldehyde (31.25 $IU.ml^{-1}$/0.26 µl $.ml^{-1}$) applied in laboratory medium Malt extract agar designed as (MEA=C_1) and its multiple concentrations (1.5MEAand 2 MEA) were then applied in orange nectar(16° Brix and pH 4.2)**.**

The combinations were, MEA =31.25/0.26 (C_1); 1.5MEA=46.8/0.39 (C_2); 2MEA =62.5/0.52 (C_3) $IU.ml^{-1}$/µl $.ml^{-1}$).

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Table 4: Combination of nisin and cinnamaldehyde * on D-values *of Alicyclobacillus acidoterrestris* spores in malt extract agar.

	Control			Cinnamaldehyde/Nisin		
	D(min)[a]	**±SD[b]**	**%Reduction.[c]**	**D(min**	**±SD[b]**	**%Reduction**
85 ºC	38.9 ^{aA}	0.55	———	24.8 ^{aD}	1.21	36%
90 ºC	15.3 ^{bB}	0.6	———	7.65 ^{bC}	0.66	50%
95 ºC	6.25 ^{cA}	0.3	———	3.8 ^{cB}	0.52	38%

a) D: Decimal reduction time ; b)Values are means± standard deviations . c) Reduction percentage.

With each column, means with the same small letter are not significantly different and each row, means with the same capital letter are not significantly differen ($p > 0.05$).All results are average of *nisin/cinnamaldehye concentration were 31.25/0.26 IU.mL^{-1}/ µ L.ml^{-1}.

Table 5: Synergitism of Nisin / Cinnamaldehyde on D-values *of Alicyclobacillus acidoterrestris* spores in orange nectar .

Combinations	**85 ºC**				**90 ºC**				95 **ºC**			
	D(min)[a]	**±SD[b]**	**R^2**	**%Red.[c]**	**D(min)**	**±SD**	**R^2**	**%Red.**	**D(min)**	**±SD**	**R^2**	**%Red.**
C_0	52.3^{aA}	0.9	0.514	———	10.8^{aB}	0.9	0.949	———	7.2^{aC}	0.8	0.966	———
C_1	42.9^{bB}	0.55	0.8181	17.9%	8.6^{bB}	1.02	0.981	20.3%	4.4^{bC}	0.76	0.991	40%
C_2	34.1^{cA}	0.94	0.5518	16.8%	5.9^{cB}	1.2	0.923	45.3%	2.3^{bcC}	0.98	0.976	59.3%
C_3	25.9^{dA}	1.02	0.7506	50.4%	5.1^{cB}	0.3	0.869	52.7%	1.1^{cC}	1.52	0.969	65.6%

a) Decimal reduction time ; b) Values are means± standard deviations . c) Reduction percentage.

With each column, means with the same small letter are not significantly different and each row, means with the same capital letter are not significantly differen ($p > 0.05$).All results are average of three trials. C_0: 0 ; C_1: 31.25/0.26 ; C_2 :46.8/0.39 ; C_3:62.5/0.52 IU.mL^{-1}/ µ L.ml

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Data presented in **Table (5**) show the effect of these combinations on the D values of activated spores of *A .acidoterrestris* in orange nectar. It is to be noted the great increase in the D values of the spores in orange nectar comparing with D-values obtained in MEA as a media. Where, exposure of the activated spores to increased concentrations of this combination at the same temperature resulted in an extra reduction in D values. The D-values decrease gradually 42.9 ;34.1 ;25.9 min with a highest reduction percentage (50.4%)at 85°C .This result was explained by Silva and Silva (22) who stated that the D-values measured in real fruit systems, were higher than those predicted by the malt extract broth model. They attributed this change in the D values to the presence of other constituents in the fruit products and not in the MEB that have effects different from those orange nectar during of fructose used for the model generation and that might increase the heat resistance of the spores .Moreover, Ceviz *et al* (23) has stated that temperature has been found to be the most important factor having effects on D-value, which is the main indicator of the thermal resistance of *A. acidoterrestris* spores among SS, pH and temperature values .

Effect of nisin and cinnamaldehyde on the outgrowth of activated *A.acidoterrestris* spores inoculated in orange nectar during storage periode.

The concentration of nisin /cinnamaldehyde C_2 (46.8 $IU.ml^{-1}$/0.39 $\mu l.ml^{-1}$) was selected to be applied individually and in combination for comparing the synergistic effect to extent the shelf-life of orange nectar.

Nisin and cinnamaldehyde combination on *A.acidoterrestris* spores in pasteurized orange nectar during storage at 25 and 45 °C.

Data recorded in Table**(6**) summarize the effect nis/CNMA on the outgrowth of activated Alicyclobacillus *acidoterrestris* spores ($LogCFU.ml^{-1}$) in pasteurized orange nectar during storage at 25 °C .

It is clearly noticed that in orange nectar not supplemented with any antimicrobial, the initial numbers of germinated spores showed exponential growth during storage period.

Table 6: Inhibition of outgrowth of *Alicyclobacillus acidoterrestris* spores ($LogCFU.ml^{-1}$) by nisin /CNMA in pasteurized orange nectar during storage at 25 ºC.

Time(in days)	Antimicrobials			
	Control	Nisin	Cinna	Nis/Cin
0	4.69	4.69	4.69	4.69
3	4.47	2.02	———	———
6	4.47	———	———	———
9	4.47	———	———	———
12	5.02	———	———	———
15	5.11	———	———	———
18	5.90	1.65	———	———
21	5.84	2.07	———	———
24	6.00	2.07	———	———
27	6.04	2.11	———	———
30	6.02	2.39	———	———
33	6.13	2.60	1.30	———
36	6.80	3.77	1.30	———
39	6.80	3.83	1.54	———
42	7.04	3.86	2.14	———
45	8.00	4.00	3.00	———
48	8.02	4.17	3.60	1.59
51	8.50	4.69	3.85	1.60

The addition of 46.8 $AU.ml^{-1}$ of nisin an obvious decrease in viability extent to the 3rd day after which the growth was totally inhibited, and no viable cells were detected for 15 days, followed by a few viable cells ranged between 1.65 and 2.07 log CFU. ml^{-1} form the 18th to 24th day of storage , apparently which may be originated from a very low number of survivors . According to the Egyptian organization of standardization (E.S) and quality (24) for the non –carbonated sweeten drinks for fruit nectars that the total viable count

should not exceed 100 cells /ml (equivalent to 2 log CFU.ml^{-1}),this concentration extent the shelf –life of nectar to the 18 th day.

By the addition of cinnamaldehyde individually, the degree of the complete inhibition extended to the 30th day , while the total viable count still within the legal permitted limits by E.S till the 39th day. On the other hand, the combination of both antimicrobial extent the total inhibition to reach the 45 th day, this result confirmed our previous findings of synergistic effect .Where, nisin effect could be greatly potentiated by the presence of low concentration of cinnamaldehyde . The growth of Alicyclobacillus cells in pasteurized nectar may be explained by Jensen and Whitfield (25) who stated that a factor affecting halophenol (2,6,-DCP have been linked to the production in fruit juice by *A .acidoterrestris*)production is the headspace of the packging of the fruit juice . A larger headspace results in increase in the growth rate of *A. acidoterretris* and thus an increase in the rate of halophenol production .

The concept of using a variety of antimicrobials to achieve optimal antimicrobial activity is called the multiple hurdle approach (26). Moreover, the concept of synergistic between nisin and cinnamaldehyde was explained by Yamazaki et al (27)**,** hence combined antimicrobials with different modes or sites of activity achieve higher antimicrobial efficiency while significantly diminishing the resistance that microorganisms will develop. The more varied these ‘‘hurdles’’ are, the more effective they are and the lower of target cells surviving the treatment and developing resistance.

In the present results the nisin, individually, showed sporostatic action against the spores of *A. acidoterrestris* rather than sporicidal . The same trend was obtained in the results of (27) who stated the inhibitory effects of nisin on the growth of the thermoacidophilic spoilage .In the contrary, (18) described that the nisin action against spores was sporicidal rather than sporostatic, where it inhibit the germination process at the stage of preemergent swelling .

Results in (**Table 7**) represent the effect of nisin and cinnnamaldehyde on the outgrowth of activated *Alicyclobacillus acidoterrestris* spores (LogCFU.ml-1) in pasteurized orange nectar during storage at 45 °C.

Table 7: Inhibition of outgrowth of *Alicyclobacillus acidoterrestris* spores (LogCFU.ml^{-1}) by nisin /CNMA in pasteurized orange nectar during storage at 45 ºC.

Time(in days)	Antimicrobials			
	Control	Nisin	Cinna	Nis/Cin
0	4.84	4.84	4.84	4.84
3	4.86	———	———	———
6	5.00	———	———	———
9	5.11	———	———	———
12	5.11	1.35-	———	———
15	5.69	1.30	———	———
18	5.85	1.39	———	———
21	6.00	1.67	———	———
24	6.04	1.81	1.26	———
27	6.13	2.14	1.47	———
30	6.23	2.39	1.54	———
33	6.45	3.0	1.77	———
36	7.14	3.87	2.00	1.38
39	7.5	4.69	3.04	1.90
42	7.57	4.84	3.66	1.95
45	7.80	5.02	4.17	2.11
48	7.85	5.20	4.50	2.75
51	8.02	5.90	4.50	2.84

This temperature was chosen to determine growth patterns of *A. acidoterrestris* under optimum growth conditions and to determine the length of time until the organism reaches a concentration that is associated with taint. An exponential increase in the germination of the activated spores in absence of antimicrobial this is probably to the increase in temperature to the optimal limits conditions of growth. A

general decrease in both antimicrobial potentialities individually with a distinguishable reduction in the complete inhibition during storage. By exposure of the orange nectar to nisin , at 45° C , a complete inhibitory effect was seen to the 9 th days, this period extended to the 21 th day with the treatment by cinnamaldehyde while, their combination showed the highest complete inhibition period of to 33 th day, suggesting to be a promising way to extend the shelf life .While as, the total viable activated spores showed an obvious gradual increase within the legal permitted limits by (24) till the 24th ,36th ,42 th day for the nisin ,cinnamaldehyde, and their combination,respectively.

The appearance of *A. acidoterrestris* cells in pasteurized nectar is demonstrated by (28) who stated that the amount of headspace has a significant effect on growth of vegetative cells and spores of *A. acidoterrestris* at 35 °C. Intermittent shaking before sampling increased growth and therefore probable detection rates at 30°C. Agitating containers and sampling from several areas within containers is therefore recommended for determining whether *A. acidoterrestris* is present or absent from stored juice, especially in large containers.

Also, Spinelli *et al* [29] has examined the effect of storage temperature on *A. acidoterrestris* growth in hot-filled orange juice. It was observed that *A. acidoterrestris* predicted growth parameters were significantly influenced ($P < 0.05$) either by inoculum level or cooling and storage conditions. Therefore, hot-filled orange juice should be stored at or below 20°C to avoid spoilage by this microorganism.

Nisin and cinnamaldehyde combination on *A.acidoterrestris* spores in unpasteurized orange nectar during storage at 25 and 45 °C.

The obtained results in **Table (8**) describe the effect of nisin and cinnamaldehde individually and in combination on the outgrowth of activated *Alicyclobacillus acidoterrestris* spores (LogCFU.ml^{-1}) in unpasteurized orange nectar during storage at 25 °C. It is to be noted that the potential of antimicrobial inhibitory effect of both antimicrobials was greatly affected compared to the unpasteurized nectar.

Where an obvious shortening in the complete inhibition period till the 9 th ;18 th ;27 th day for nisin , cinnamaldehyde and nis/cinna combination, respectively. On the other hand, according to Eyptian Organization of Standardization and Quantity [24], the safety period for the nectar was recorded to 42th day in case of combination ,where the recovered cells did not exceed 2 log CFU.ml^{-1} providing additional evidence of the effectiveness of synergism between these antimicrobials.

Table 8: Inhibition of outgrowth of *Alicyclobacillus acidoterrestris* spores (LogCFU.ml^{-1}) by nisin /CNMA in unpasteurized orange nectar during storage at 25 ºC.

Time(days)	Antimicrobials			
	Control	Nisin	Cinna	Nis/Cin
0	4.17	4.17	4.17	4.17
3	4.13	———	———	———
6	4.84	———	———	———
9	5.06	———	———	———
12	5.17	1.54	———	———
15	5.20	2.39	———	———
18	5.90	2.47	———	———
21	6.04	2.77	1.29	———
24	6.20	2.60	1.81	———
27	6.46	3.17	1.95	———
30	6.85	3.22	2.00	1.54
33	7.10	3.64	2.14	1.74
36	7.23	3.69	2.47	1.68
39	7.64	3.85	2.64	1.65
42	7.60	4.08	3.30	1.93
45	7.85	4.69	3.81	2.38
48	8.15	4.90	3.74	2.47
51	8.35	5.11	4.06	2.55

The role of interference of cinnamaldehyde with energy generation in the mechanism of action, the cellular and extracellular ATP levels of cells in 4-2 hydroxylethyl-1-piperazineethanesulfonic acid (HEPES) buffer at 20°C. This is because cells that are unable to reproduce or alter their metabolism [30].

In addition, Pathanibul *et al* [31] has demonstrated the effectiveness of nisin as non thermal technology for the inactivation of microorganisms as *Escherichia coli* and *listeria innocua* , in fruit and vegetables juices, and the synergism of nisin with the high pressure homogenization in apple and carrot juice .

Moreover, Periago and Moezelaar [32] have stated that the combined effect of nisin and carvacrol was found to be significantly different at pH 7.0 and 5.75. Also, at this low temperature, a synergistic effect between nisin and carvacrol on *B. cereus* was observed at the pHs tested.

The obtained results from **Table (9)** represent the nisin and cinnaldehyde on the outgrowth of activated Alicyclobacillus *acidoterrestris* spores (LogCFU.ml^{-1}) in unpasteurized orange nectar during storage at 45 ºC. . Once more, by increasing the temperature of storage to a nearest point of the optimal condition for spore germination and growth a linear distinguishable increase in recovered CFU in the non supplemented nectar with antimicrobials till the last storage period. While as, the complete inhibition periods was the lowest out of all the tested cases.

Table 9: Inhibition of outgrowth of *Alicyclobacillus acidoterrestris* spores (LogCFU.ml^{-1}) by nisin /CNMA in unpasteurized orange nectar during storage at 45 ºC.

Time in Days	Antimicrobials			
	Control	Nisin	Cinna	Nis/Cin
0	4.47	4.47	4.47	4.47
3	4.58	———	———	———
6	4.75	———	———	———
9	4.88	1.60	———	———
12	5.05	1.63	———	———
15	5.60	1.77	———	———
18	5.55	2.11	1.48	———
21	5.90	2.14	1.84	———
24	6.00	2.20	1.95	1.63
27	6.04	3.30	2.14	1.65
30	6.80	3.81	2.25	1.77
33	6.95	3.82	2.47	2.02
36	7.50	4.07	3.82	2.14
39	7.90	4.20	4.05	2.36
42	7.85	4.25	4.75	2.84
45	8.05	5.10	4.70	3.03
48	8.20	6.02	5.01	3.17
51	8.59	6.20	5.11	3.82

Thus,it were the 6 th ; 15 th ;21th day , for nisin, cinnamaldehyde and their combination, respectively. Where the maximum yield of complete inhibition was recorded by the combination confirming the synergistic effect between these tested antimicrbials .By adding of nisin ,the sizes of surviving population reappeared and increase slowly from1.60 to 1.63 log CFU/ml .By exposure of the nectar inoculated by the activated to cinnamaldehyde the proliferation restated and increase from 1.48 to 1.95 log CFU/ml at the 24 th day. A larger increase within the legal permitted period of E.S. by combining both antimicrobials, thus the recovered viable CFU was 1.63.to 1.77 from the complete inhibition to the 30 th day.

It is to be noticed that the more the temperature increase the more the effectiveness of both antimicrobials decrease this phenomenon was explained by at lower temperature the proportion of unsaturated fatty acyl chains of the lipids is increased resulting in a higher membrane fluidity ,which could facilitate the incorporation lipophilic compounds as nisin or essential oil[33].

The combinination of nisin with plant essential oils, the restrictions in the use of nisin as a food preservative might be overcome and the range of applications could be expanded. Since both compounds act on the cytoplasmic membrane, an additive or synergistic effect could be expected, and a lower dosage of both compounds would be necessary to cause an inhibitory effect as reported by [34].

CONCLUSIONS

The combination of nisin and cinnamaldehyde at concentration (46.8. $IU.ml^{-1}$/0.0.39 $\mu l.ml^{-1}$))have a synergistic effect and represent a promising base to be used as food natural food preservatives to control the growth of *A. acidoterrestris* and prolongation of the shelf –life to 21 days of fresh unpasteurized orange nectar at 45°C and for 33 days in pasteurized nectar at the same temperature.

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