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Effect of Nisin on the Physical and Chemical Characteristics of Apple Juice and the Control of *Alicyclobacillus acidoterrestris*

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Abstract

Apples are sold as fresh fruits on the national and international markets, and sub-standard apples are often used for juice production. The juice can be contaminated by *Alicyclobacillus acidoterrestris*, a spore-producing thermoacidophilic bacterium that is resistant to the pasteurization process. *Alicyclobacillus acidoterrestris* causes deterioration of the juice and alters its taste, and the use of nisin is one of the options available to combat contamination by the bacterium. In this work, nisin was tested at concentrations of 5, 10, 25, 50, and 100 UI/mL for inhibition of germination of the bacterial spores, and at a concentration of 10 UI/mL for evaluation of changes in the physicochemical characteristics of apple juice (pH, colour, vitamin C content, and concentration of phenols). The results showed that nisin was able to significantly decrease spore germination, without changing the characteristics of the apple juice.

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Keywords: *Alicyclobacillus acidoterrestris;* apple; juice; nisin; spores.

1. Introduction

Apples are grown in temperate climate zones and sold fresh on national and international markets [1].In Brazil, there are few apple orchards dedicated to industrial-scale production of juice, and the part of the harvest considered unsuitable for the fresh market (15-30% of the total apple production) is used for processing, mainly in the form of juice. Around 90% of the apple juice produced in the country is in the form of concentrate destined for export, especially to the United States where the concentrate is used to produce reconstituted juice and products for children [2].

One of the characteristics of apples is that the fruit is liable to infection by thermoresistant microorganisms such as *Alicyclobacillus* spp., and contamination of apple juice occurs when the microbes survive the pasteurization process [3]. Species of the genus *Alicyclobacillus* are thermoacidophilic organisms that under favourable environmental conditions produce thermoresistant spores. The spores then grow within the packaged juice, causing changes in its flavour and odour[4, 5].

The contamination of juice with*A. acidoterrestris* usually occurs when there is inadequate previous cleaning of the fruit, resulting in the transfer of the organism to the juice. In 1982, there was large-scale deterioration of apple juice that had been packaged aseptically in Germany, which alerted the juice industry to the potential of *Alicyclobacillus acidoterrestris*to cause contamination [6]. The main technique used to reduce the quantity of microorganisms in juice is pasteurization [7]. However, this procedure alone is no longer considered suitable in the case of *Alicyclobacillus*,due to the thermoresistance of the organism. Certain bacteriocins, such as nisin, can be used together with pasteurization in order to inhibit the growth of these microorganisms.

Nisin is an antimicrobial peptide extracted from *Lactococcuslactis*that shows a wide range of inhibitory effects against gram-positive microorganisms. The compound is highly stable at low pH as well as at high temperatures [7]. It is less effective against gram-negative bacteria, yeasts, and other fungi [8]. The fact that nisin is a non-toxic natural preservative enables it to be used in the food industry. The present work investigates the ability of nisin to inhibit the germination of spores of *Alicyclobacillus acidoterrestris* in apple juices, as well as its effect on physicochemical characteristics of the juice.

2. Materials and Methods

2.1 Organism and culture media

The Alicyclobacillus acidoterrestris cell line, isolated from apple juice, was obtained from a commercial culture collection.

The medium used for sporulation was *Bacillus acidoterrestris* agar (BAT) containing yeast extract (2 g), glucose (5 g), calcium chloride (0.2566 g), magnesium sulphate (0.5 g), ammonium sulphate (0.2 g), potassium dihydrogen phosphate (3 g), zinc sulphate ($1.8x10^{-4}$g),copper sulphate ($1.6x10^{-4}$g), manganese sulphate($1.5x10^{-}$

^{4}g),cobalt chloride ($1.5x10^{-5}$g),boric acid($1.0x10^{-4}$g), sodium molybdate ($3.0x10^{-4}$g), and AgarAgar (18 g). The medium was made up in a volume of 1000 mL of distilled water, and the final pH was adjusted to 4.0 [9].

The enrichment broth was prepared using peptone (5 g), glucose (10 g), yeast extract (2.5 g), Tween80 (1 g), AgarAgar(15 g), and distilled water (1000 mL). The medium was sterilized at 121 °C for 15 min [9].

2.2 Preparation of the nisin bacteriocin

A stock nisin solution was prepared by diluting commercial Nisaplin (Aplin& Barrett, UK) in 200 mL of 0.02 M HCl, and was then sterilized by passage through a Millipore membrane (0.22µm porosity).

2.3 Production of spores of Alicyclobacillus acidoterrestris

For spore production, the *Alicyclobacillus acidoterrestris* isolates were grown in the enrichment broth at 43ºC for 24 h. The growth of the spores was confirmed using the malachite green colour test, which is specific for spores. In order to achieve a high degree of sporulation, 100µLof broth with spores was inoculated onto a Petri dish with BAT medium, and maintained at 44ºC for 72 h. After growth, all the colonies were transferred to a test tube containing 10 mL of 0.85% sterile saline solution. The cultures were centrifuged at 2000 rpm for 10 min and the supernatant was discarded. The centrifuged material was then re suspended in 10 mL of 0.85% sterile saline solution [7]. The suspension of spores was centrifuged three times and the supernatant was discarded. The spores were once again re suspended in 10 mL of 0.85% sterile saline solution. The suspension was maintained in a water bath at 80 ºC for 10 min in order to inactivate the vegetative cells and activate the spores. The sample suspensions were stored at 4ºC for no longer than one week before use [10].

2.4 Effect of nisin on vegetative cells inoculated into BAT culture medium

Alicyclobacillus acidoterrestris was incubated at 43 ºC in BAT culture medium, using a cell concentration of 10^{3} CFU/mL and different nisin concentrations(5, 10, 25, 50, and 100 UI/mL). The second method employed was diffusion in wells, as described by Harris et al. (11), with modifications. For each test, 5-10 UI of nisin were placed in holes (wells) made in BAT agar in Petri dishes that had been previously inoculated with *Alicyclobacillus acidoterrestris.* The Petri dishes were incubated at 43°C for 48 h, followed by examination of the formation of spore inhibition halos and comparison with a positive control.

2.5 Evaluation of the physicochemical characteristics of apple juice after use of nisin preservative

2.5.1 Samples

The physicochemical characteristics of the juice were evaluated using four types of sample: A - 25 mL of apple juice; AS - 25 mL of apple juice with solution of spores (10^{1}); ASN - 25 mL of apple juice with solution of spores (10^{1}) plus nisin (10 UI/mL); AN - 25 mL of apple juice with nisin (10 UI/mL). The samples were kept in a refrigerator (at 2-8^{o}C) and at ambient temperature (25^{o}C), and analyses were performed after 0, 7, 14, 21, and

28 days. The different juices were also inoculated onto a BAT agar plate and incubated at 43 ºC for 3 days in order to evaluate microbial growth.

2.5.2 Measurement of pH

The pH of the samples was determined using a Quimis Model Q488AS potentiometer that had been previously calibrated using pH 4 and pH 7 buffer solutions immediately prior to measurements of the samples.

2.5.3 Spectrophotometric colour analysis

The samples were diluted (1:10) in distilled water, followed by measurements of absorbance at 420 and 520 nm. Thecolour valuewas calculated by dividing the absorbance at 420 nm by the absorbance at 520 nm[12].

2.5.4 Vitamin C content

The concentration of vitamin C in the apple juice was determined based on the reducing ability of the vitamin, by titration using 2,6-dichlorophenol-indophenol. The dye solution was first standardized by titration against an ascorbic acid solution of known concentration (100 mg/dL). The titre of dye solution obtained was then used to calculate the vitamin C content as follows: Vitamin C (mg/mL) = 2 mg/mL x VA (mL)/VP (mL), where 2 mg/mL corresponds to the amount of vitamin C contained in 2 mL of the standard, VP (mL) is the volume of dye solution required to titrate the ascorbic acid standard solution, and VA (mL) is the volume of dye solution required to titrate the apple juice.

2.5.5 Determination of total phenols

Quantification of phenolic compounds was performed using the Folin-Ciocalteau method [13]. This technique is based on the formation of a blue complex due to reduction of the Folin-Ciocalteau reagent by phenolic compounds. For the colorimetric reaction, an aliquot of 0.5 mL of an ethanolic solution of the juice (diluted 1:20) was added to 2.5 mL of an aqueous 10% solution of the Folin-Ciocalteau reagent and 1.5 mL of 20% sodium carbonate. The mixture was incubated in the dark for 2 h at ambient temperature, and the absorbance was measured at 760 nm, using the blank as a reference.

3. Results and Discussion

3.1 Action of nisin on Alicyclobacillus acidoterrestris

The use of nisin resulted in significant inhibition of germination of the spores of *A. acidoterrestris*, compared to the samples inoculated without nisin (Table 1). In the absence of nisin, the cell count was 6.17 CFU/mL, while in the presence of nisin it was 5.06 CFU/mL. The degree of inhibition of germination was the same using 50 and 100 UI/mL of nisin, so the lower concentration could be used to obtain the same effect.

The results of the halo inhibition tests showed that in the absence of nisin, there was no inhibition of spore germination, with uniform growth of the microorganisms on the plate. Use of nisin at concentrations of 5 and 10

UI/mL resulted in the formation of significant inhibition halos. The inhibition was greatest for the higher nisin concentration (10 UI/mL), for which ahalo of 29mm was obtained.

Table 1. Germination of spores of *A. acidoterrestris* (CFU/mL) in BAT culture medium, in the presence of nisin, after incubation at 43° C for 48 h (initial inoculum of 3.0 log CFU/mL).

Conservative	Log UFC/ml	Diâmetro da zona de inibição (mm)
No coservative	6.17± 0.52	No inhibition
Nisin5 UI/ml	5.40 ± 0.32*	23 ± 1.5
Nisin 10 UI/ml	5.34 ± 0.36*	29 ± 2
Nisin 25 UI/ml	5.18 ± 0.37*	----------
Nisin 50 UI/ml	5.06 ± 0.34*	----------
Nisin 100 UI/ml	5.06 ± 0.34*	----------

*Significant difference compared to control without conservative $p<0.05$

The germination of spores of *A. acidoterrestris* in apple juice stored at ambient temperature is shown in Table 2, from which it can be seen that nisin provided inhibition of germination during up to 28 days of storage. The sample of juice enriched with spores (positive control) showed growth of the microorganism throughout the period, with cell counts of between 2.41 and 2.48 CFU/mL.

Table 2. Germination of spores of *A. acidoterrestris* (CFU/mL) in concentrated apple juice, at ambient temperature, in the presence of 100 UI/mL of nisin (initial inoculum of 1.0 log CFU/mL)

	Days			
Sample	7	14	21	28
Apple juice (A)	NG	NG	NG	NG
Apple juice + spores (AS)	2.41	2.47	2.41	2.48
Apple juice + nisin (AN)	NG	NG	NG	NG
Apple juice + spores + nisin (ASN)	NG	NG	NG	NG

NG = no bacterialgrowth

The results of the tests of spore germination in the juice samples kept refrigerated and at ambient temperature were similar (Table 3). The juice with spores alone showed microbial growth throughout the period, while the samples containing nisin showed no growth, indicating the effectiveness of nisin against the spores of *A. acidoterrestris.*

In earlier work, Peñaet al. [7] investigated the use of nisin in juice at concentrations of 0, 50, 75,and 100 UI/mL, and pasteurization temperatures of 92, 95, 98, and 102 °C. The results showed that when nisin was added to the juice, the thermal resistance of the microorganism (at a given temperature) decreased in line with increase of the

nisin concentration. The microbial resistance was affected first by the temperature and then by the nisin concentration. The addition of nisin therefore offers a means of reducing the rigour of thermal treatment to combat*A. acidoterrestris*, as shown by the results obtained here, where the samples were not submitted to thermal inactivation of the organism.

Table 3.Germination of spores of *A. acidoterrestris* (CFU/mL) in concentrated apple juice kept refrigerated (at 2-8 ºC), in the presence of 10 UI/mL of nisin (initial inoculum of 1.0 log CFU/mL).

	Days			
Sample	7	14	21	28
Apple juice (A)	NG	NG	NG	NG
Apple juice + spores (AS)	2.35	2.40	2.69	2.61
Apple juice + nisin (AN)	NG	NG	NG	NG
Apple juice + spores + nisin (ASN)	NG	NG	NG	NG

NG = no bacterialgrowth

Similar to the effects of nisin demonstrated in the present work, other studies have reported the benefits of using the bacteriocinbificin C6165 against vegetative cells of *A. acidoterrestris* in apple juice. However, the effect of bificin C6165 was achieved at a low pH (3.5) and a temperature of 45 ºC [14]. The present findings indicate that nisin is more effective than bificin C6165, as it was able to inhibit the germination of*A. acidoterrestris* in apple juice maintained both refrigerated and at ambient temperature, for up to 28 days.

In work by [15], saponins were used for inhibition of the spores of *Alicyclobacillus* spp. After 2 h of incubation with a commercial saponin, there was a reduction of up to 0.5 log cycles of *Alicyclobacillus* spp., and a statistically significant reduction in the number of spores was obtained after 5 days of incubation with commercial saponin at 45 °C. The most effective method for inactivation of*A. Acidoterrestris* using commercial saponin was achieved with concentrations of 300 and 400 mg/L, after 10 days, and 500 mg/L, after 5 days.

According [16], the presence of nisin did not significantly influence the inactivation of *Salmonella typhimurium* or *E. coli*. In tests using diffusion in wells, [11] found that nisin was unable to inhibit the growth of *Listeria monocytogenes*, mainly due to proteolytic degradation of the inhibitor. However, the present work showed that nisin provided effective inhibition of *A. acidoterrestris*, with the formation of halos up to 26 mm in diameter.

3.2 Physical and chemical characteristics of apple juice incubated under different conditions

The pH of the apple juice varied between 3.40 and 3.72 (Table 4), in agreement with the measurements made by Vieira [17] for apple pulp, where the pH range was3.40-4.16. *A. acidoterrestris* is able to grow at pH between 2.5 and 6.0, although the optimum pH is between 3.5 and 4.0 [4]. The fact that the pH measured here was close to this range provides further support for the ability of nisin to inhibit the growth of this microorganism.

Table 4. Effect of nisin and spores of *A. acidoterrestris* on the pH of concentrated apple juice kept at ambient temperature (AT) or refrigerated (R). Apple juice (A); Apple juice + spores (10^1) (AS); Apple juice + nisin (10 UI/mL) (AN); Apple juice + spores (10^1) + nisin (10 UI/mL) (ASN).

Sample	Days								
	0	7		14		21		28	
		AT	R	AT	R	AT	R	AT	R
A	3.59±0.01	3.59±0.02	3.60±0.01	3.72±0.01	3.71±0.04	3.69±0.01	3.71±0.05	3.57±0.02	3.52±0.04
AS	3.60±0.01	3.62±0.01	3.58±0.02	3.71±0.01	3.72±0.03	3.69±0.02	3.71±0.04	3.56±0.01	3.53±0.04
AN	3.59±0.01	3.59±0.01	3.55±0.03	3.69±0.02	3.70±0.03	3.70±0.01	3.70±0.03	3.47±0.03	3.52±0.06
ASN	3.60±0.01	3.59±0.02	3.56±0.03	3.69±0.01	3.71±0.04	3.70±0.01	3.70±0.04	3.37±0.03	3.51±0.05

Table 5 gives the different intensities of colour of the apple juice incubated under the different conditions, with or without spores of*A.acidoterrestris.* The intensity of colour gradually increased over the course of the incubation period, both under refrigeration and at ambient temperature. The colour became increasingly yellow, and was dark yellow in the final week. The change in colour was associated with decreased levels of vitamin C and total phenols, due to oxidation of the juice. At ambient temperature, sample SEN showed the least darkening, while under refrigeration the smallest change was shown by sample SN.

Analysis of the vitamin C content (Table 6) revealed a progressive decrease, with the concentration for sample SEN decreasing from an initial value of 2.65 mg/mL to concentrations of 0.528 mg/mL (ambient temperature) and 0.53 mg/mL (refrigeration) after 28 days. There was an approximately 50% decrease in the vitamin C content after 21 days. In work using orange juice, [18] found that there was a rapid degradation of vitamin C during the initial phase of storage, followed by slower loss, which was attributed mainly to the oxygen content of the juice, especially during the early storage period.

Table 5. Effect of nisin and spores of *A. acidoterrestris* on the colour of concentrated apple juice kept at ambient temperature (AT) or refrigerated (R). Apple juice (A); Apple juice + spores (10^1) (AS); Apple juice + nisin (10 UI/mL) (AN); Apple juice + spores (10^1) + nisin (10 UI/mL) (ASN).

Sample	Days								
	0	7		14		21		28	
		AT	R	AT	R	AT	R	AT	R
A	2.3±0.006	2.3±0.005	2.29±0.005	3.1±0.005*	5.02±0.002*	4.27±0.002*	4.26±0.003*	4.86±0.004*	5.02±0.007*
AS	2.45±0.005	2.6±0.004	2.26±0.004	4.3±0.010*	4.7±0.004*	4.16±0.003*	4.78±0.005*	4.13±0.002*	4.76±0.006*
AN	2.35±0.006	2.32±0.003	2.16±0.002	2.72±0.004	2.72±0.005*	4.32±0.005*	4.31±0.007*	4.3±0.005*	3.71±0.002*
ASN	2.25±0.004	2.18±0.003	2.10±0.001	3.85±0.004*	3.85±0.006*	4.14±0.002*	4.56±0.008*	4.08±0.004*	4.41±0.004*

* Significantdifference fromtimezerop<0.05

Table 6. Effect of nisin and spores of *A. acidoterrestris* on the concentration of vitamin C (mg/mL) in concentrated apple juice kept at ambient temperature (AT) or refrigerated (R). Apple juice (A); Apple juice + spores (10^1) (AS); Apple juice + nisin (10 UI/mL) (AN); Apple juice + spores (10^1) + nisin (10 UI/mL) (ASN).

Sample	Days								
	0	7		14		21		28	
		AT	R	AT	R	AT	R	AT	R
A	2.54±0.06	3.4±0.10	2.46±0.05	1.16±0.05*	0.725±0.15*	0.875±0.12*	0.62±0.03*	0.752±0.04*	0.50±0.07*
AS	2.63±0.10	2.7±0.04	2.7±0.04	1.55±0.10*	0.61±0.04*	0.69±0.08*	0.60±0.12*	0.55±0.09*	0.59±0.11*
AN	2.58±0.05	2.23±0.12	2.93±0.02	0.85±0.04*	0.775±0.14*	0.81±0.05*	0.60±0.07*	0.64±0.05*	0.49±0.08*
ASN	2.65±0.06	1.64±0.15	2.35±0.04	0.95±0.05*	0.575±0.12*	0.89±0.06*	0.58±0.08*	0.528±0.09*	0.53±0.07*

* Significantdifference fromtimezerop<0.05

The content of total phenols (Table 7) also decreased during storage at room temperature and under refrigeration. In work by Nogueira and colleagues [2], who analyzed total phenols in juice of the apple varieties Fuji, Gala, and Golden Delicious, losses of phenols contributed to oxidation of the juice, although significant differences ($p<0.05$) were observed between the three apple varieties. [19] reported that the enzymatic oxidation darkening reaction was the main cause of loss of phenolic compounds and reduced antioxidant activity in apple extracts.

Table 7. Effect of nisin and spores of *A. acidoterrestris* on the content of total phenols (mg/mL of gallic acid equivalents) in concentrated apple juice kept at ambient temperature (AT) or refrigerated (R). Apple juice (A); Apple juice + spores (10^1) (AS); Apple juice + nisin (10 UI/mL) (AN); Apple juice + spores (10^1) + nisin (10 UI/mL) (ASN).

Sample	Days								
	0	7		14		21		28	
		AT	R	AT	R	AT	R	AT	R
A	87±1.15	88±1.8	87±3.5	86±1.6	78±1.8	86±2.7	86±2.6	79±3.0	86±4.7
AS	87±1.16	84±2.7	80±1.6	79±1.0	80±2.8	83±4.6	81±2.9	80±4.1	81±2.2
AN	83±1.2	84±3.2	72±2.2	78±2.5	78±1.8	80±2.7	84±3.4	77±3.3	83±4.5
ASN	86±1.5	86±2.5	80±2.7	78±2.6	80±3.5	80±3.3	80±3.9	82±1.7	79±2.8

The physicochemical characteristics of apple juice are directly related to the degree of oxidation, and in the enzymatic darkening reaction the concentration of phenols is altered due to the action of the polyphenoloxidase enzyme[20]. According to Nogueira and colleagues [2], the phenolic compounds are of commercial interest due to their influence on sensory characteristics as well as their antioxidant capacity (which protects against the effects of oxidative stress).The addition of antioxidants can be used to block enzymatic oxidation and/or provide

reducing capacity. Vitamin C is an antioxidant used to prevent darkening in fruits because the presence of this acid reduces quinine compounds to their phenolic forms [10], hence affecting the phenol content.

4. Conclusions

The present findings support the use of nisin to inhibit germination of the spores of*Alicyclobacillus acidoterrestris*, a causative agent of the deterioration of apple juice. The use of nisin did not significantly affect the physicochemical characteristics of the juice, including pH, total phenols content, vitamin C concentration, and colour. It can therefore be concluded that nisin can be used together with pasteurization to provide better control of the germination of *A. acidoterrestris*spores as well as the growth of other thermoresistant microorganisms in apple juice.

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