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ALICYCLOBACILLUS ACIDOTERRESTRIS:FORMAÇÃO DE BIOFILMES E INIBIÇÃO DO SEU CRESCIMENTO EM SUCO DE LARANJA POR NANOPARTÍCULAS DE CURCUMINA

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TATIANE VIANA DUTRA

"*ALICYCLOBACILLUS ACIDOTERRESTRIS*: FORMAÇÃO DE BIOFILMES E INIBIÇÃO DO SEU CRESCIMENTO EM SUCO DE LARANJA POR NANOPARTÍCULAS DE CURCUMINA"

Tese apresentada à Universidade Estadual de Maringá, como parte das exigências do Programa de Pós-graduação em Ciência de Alimentos, para obtenção do grau de Doutor em Ciência de Alimentos.

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BIOGRAFIA

Tatiane Viana Dutra nasceu em 07 de janeiro de 1994, na cidade de Guaraçaí-SP; possui graduação em Engenharia de Alimentos pela Universidade Tecnológica Federal do Paraná campus Campo Mourão (UTFPR-CM), mestrado em Ciência de Alimentos pela Universidade Estadual de Maringá (UEM). Apresenta experiência na área de segurança, qualidade e microbiologia de alimentos, atuando em temas como formação de biofilmes em indústrias alimentícias, controle microbiano, atividades antimicrobianas, extratos naturais e óleos essenciais com propriedades antimicrobianas, desenvolvimento e análise de nanopartículas, no combate a bactérias deteriorantes e patogênicas.

Dedico

A minha mãe, Maria Helena, em
memória ao meu pai, Luiz Carlos,
e ao meu noivo, Renan,
pelo apoio, suporte e motivação!

APRESENTAÇÃO

Esta tese está apresentada na forma de dois artigos científicos.

1. Tatiane Viana Dutra; Daniela Biral do Prado; Márcia Maria dos Anjos; Miguel Machinski Junior; Jane Martha Graton Mikcha; Benício Alves de Abreu Filho. Contribution of environmental factors in the formation of biofilms by *Alicyclobacillus acidoterrestris* on surfaces of the orange juice industry. Ciência Rural. Accepted: 14 December 2019. https://doi.org/10.1590/0103-8478cr20190790

2. Tatiane Viana Dutra; Jéssica Lima de Menezes; Amanda Gouveia Mizuta; Anielle de Oliveira; Thaysa Fernandes Moya Moreira; Lillian Barros; Filipa Mandim; Carla Pereira; Odinei Hess Gonçalves; Fernanda Vitória Leimann; Jane Martha Graton Mikcha; Miguel Machinski Junior; Benício Alves de Abreu Filho. Use of nanoencapsulated curcumin against vegetative cells and spores of *Alicyclobacillus* spp. in industrialized orange juice. International Journal of Food Microbiology. Accepted: 13 October 2021. https://doi.org/10.1016/j.ijfoodmicro.2021.109442

GENERAL ABSTRACT

INTRODUCTION

Alicyclobacillus spp. are Gram-positive, spore-forming bacillus with the ability to adhere to surfaces and form biofilms, associated with the deterioration of acidic beverages such as orange juice. There are more than 25 species identified (Sokołowska et al., 2020), among them *A. acidoterrestris*, which is capable of producing guaiacol, which is responsible for the astringent taste in contaminated juices. This microorganism has been used as a quality parameter in the production of concentrated orange juice; therefore, it is necessary to search for alternatives for its control, mainly in Brazil, where the production and exportation of juice is of great economic importance. In this sense, the improvement of studies on its development on different surfaces is justified, in addition to the association of encapsulation techniques of natural compounds, development of nanoparticles, in order to improve its stability and potentiate its antibacterial effects.

GOALS

The objectives of this work were: evaluate the interference of the initial microbial load on the biofilm formation of *Alicyclobacillus acidoterrestris* on the surfaces of AISI 304 stainless steel and food grade natural rubber under two temperature conditions, 28 °C and 45 °C, as well as the spore load in both conditions. And evaluate the use of nano encapsulated curcumin as an antimicrobial against different strains of *Alicyclobacillus* spp. and other pathogenic bacteria, in addition to analyzing their physical characteristics, encapsulation efficiency, curcumin bioactive properties and toxicity; and as proof of concept the interference in the properties of orange juice, such as pH, color and °Brix.

MATERIAL AND METHODS

The strain *A.acidoterrestris* (CBMAI 0244T) (DSMZ 3922, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) was used for biofilm formation; the surfaces used were AISI 304 stainless steel and food grade natural rubber, both measuring 8mm x 8mm x 3mm. And the concentrated orange juice (66 °Brix) was reconstituted to 11 °Brix with sterile water.

Microbial concentrates (2 log CFU/mL and 5 log CFU/mL) were prepared at two incubation temperatures (28 and 45 °C) in reconstituted juice, and confirmed by control in BAT broth. Biofilm formation and evaluation were carried out at times 0, 4, 8, 24, 48 and 72 hours, taking into account the cleaning times of industrial

equipment; and at 28 and 45 °C, simulating the processing temperature and the ideal temperature for growth of the microorganism. To analyze the results, statistics were used using the Tukey test with a significance level of $p<0.05$.

Were also used strains of *Alicyclobacillus acidoterrestris* (CBMAI 0244T), *A. herbarius* (CBMAI 0246T), *A. acidocaldarius* subsp. *rittmanni* (CBMAI 0245T), *A. sendaiensis* (KCTC 3843), *A. hesperidum* (CBMAI 0298T), and *A. acidocaldarius* (CBMAI 0299T), in addition to *Salmonella* enterica serovar Enteritidis (ATCC 13076) and *Staphylococcus aureus* (ATCC 25923). Curcumin and PVP purchased from Sigma-Aldrich, and orange juice concentrate donated by Louis Dreyfus (LDC). Bacterial and spore suspensions were prepared with their specific growth media. Curcumin nanoparticles with PVP were obtained by solid dispersion, and characterized by FTIR, MET and DSC, in addition to evaluating their cytotoxicity and antioxidant capacity. The determination of the minimum inhibitory concentration (MIC) was carried out, as well as the minimum bactericidal concentration (MBC), in addition to the incorporation of the nanoparticles in the orange juice, with verification of their interference in pH, color and °Brix. The results were treated by ANOVA statistical analysis and Tukey's test using the Statistica 7.0 software.

RESULTS AND DISCUSSION

Regarding the results of biofilm formation, the lowest microbial load led to biofilm formation on stainless steel after 48 hours of contact at 28 °C and after 24 hours at 45 °C, while in rubber the highest formation was observed after 48 hours. contact at both temperatures. The low initial microbial load demonstrated low sporulation efficacy. Regarding the higher microbial load, biofilm formation was observed on the steel after 4 hours of contact at 28 °C and 45 °C; and in rubber such formation was observed after 8 hours of contact at 28 °C and 4 hours at 45 °C. Thus, there was a statistical difference between the temperatures, on the stainless-steel surface, in the two concentrations evaluated; for the rubber surface there was no such statistical difference.

Regarding the results of curcumin nanoparticles, its efficiency was proven, since the amounts needed to express its antibacterial activity were smaller than in its free form, against the deteriorating and pathogenic bacteria tested, such as a MIC of 125 μg/mL in Staphylococcus aureus and 62.5 μg/mL in *A. acidoterrestris*, in addition to the Fourier transform infrared (FTIR) and differential scanning

calorimetry (DSC) analyzes prove the effectiveness of the encapsulation. The images obtained by transmission electron microscopy (TEM) showed a wide range of nanoparticle sizes. The antioxidant activity assay confirmed the bioactive properties of encapsulated curcumin and also its non-cytotoxicity against four carcinoma and two non-tumor cell lines. The stability of the juice added to the nanoparticles was confirmed by maintaining their pH, color and ºBrix after 3 days of storage at 8 °C, refrigerated temperature.

CONCLUSIONS

Greater biofilm formation was observed with the highest initial microbial load on the two contact surfaces analyzed, steel and rubber, at both temperatures, but when the contact time was taken into account, there was greater formation on the rubber surface. As the formation of biofilm has already been observed after 4 hours of contact, it is necessary to perform hygiene procedures frequently. Regarding the spores, they showed greater ease of adhesion on the stainless-steel surface, with a high presence from the highest initial concentration of microorganisms.

Regarding the use of curcumin nanoparticles as antimicrobial agents, the result was satisfactory, as it proved their antimicrobial and antibacterial effect against the tested strains; in addition to the stability of the nanoparticle being confirmed by the tests performed.

Keywords: *Alicyclobacillus*, spores, orange juice, biofilm, curcumin, nanoparticles.

RESUMO GERAL

INTRODUÇÃO

Alicyclobacillus spp. são bacilos Gram-positivos, formadores de esporos, com capacidade de se aderirem a superfícies e formar biofilmes, associados a deterioração de bebidas ácidas, como suco de laranja. Há mais de 25 espécies identificadas (Sokołowska et al., 2020), dentre elas o *A. acidoterrestris*, que é capaz de produzir guaiacol, o qual é responsável pelo sabor adstringente em sucos contaminados. Este micro-organismo vem sendo utilizado como parâmetro de qualidade na produção de sucos de laranja concentrados; sendo assim necessária a busca por alternativas para seu controle, principalmente no Brasil, onde a produção e a exportação de suco são de grande relevância econômica. Nesse sentido justifica-se o aprimoramento de estudos sobre seu desenvolvimento em diferentes superfícies, além da associação de técnicas de encapsulamento de compostos naturais, desenvolvimento de nanopartículas, afim de melhorar sua estabilidade e potencializar seus efeitos antibacterianos.

OBJETIVOS

Os objetivos deste trabalho foram: avaliar a interferência da carga microbiana inicial na formação de biofilme de *Alicyclobacillus acidoterrestris* nas superfícies de aço inoxidável AISI 304 e borracha natural de qualidade alimentar em duas condições de temperatura, 28 °C e 45 °C, bem como a carga de esporos em ambas condições. E avaliar o uso da curcumina nano encapsulada como antimicrobiano frente a diferentes cepas de *Alicyclobacillus* spp. e outras bactérias patogênicas, além de analisar suas características físicas, eficiência do encapsulamento, propriedades bioativas da curcumina e toxicidade; e ainda como prova de conceito a interferência nas propriedades do suco de laranja, como pH, cor e °Brix.

MATERIAL E MÉTODOS

Foi utilizada a cepa *A.acidoterrestris* (CBMAI 0244^{T}) (DSMZ 3922, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) para formação do biofilme; as superfícies utilizadas foram o aço inoxidável AISI 304 e a borracha natural de qualidade alimentar, ambos medindo 8mm x 8mm x 3mm. E o suco de laranja concentrado (66 °Brix) foi reconstituído até 11 °Brix com água estéril.

Os concentrados microbianos (2 log CFU/mL e 5 log CFU/mL) foram preparados em duas temperaturas de incubação (28 e 45 °C) em suco reconstituído, e confirmados através do controle em caldo BAT. Procedeu-se com a formação e avaliação do biofilme nos tempos 0, 4, 8, 24, 48 e 72 horas, levando em consideração os tempos de limpeza dos

equipamentos industriais; e em 28 e 45 °C, simulando a temperatura de processamento e a temperatura ideal de crescimento do microrganismo. Para análise dos resultados utilizou-se estatística através do teste de Tukey com nível de significância $p<0,05$.

Também foram utilizadas as cepas de *Alicyclobacillus acidoterrestris* (CBMAI 0244^T), *A. herbarius* (CBMAI 0246^T), *A. acidocaldarius* subsp. *rittmanni* (CBMAI 0245^T), *A. sendaiensis* (KCTC 3843), *A. hesperidum* (CBMAI 0298^T), and *A. acidocaldarius* (CBMAI 0299^T), além de *Salmonella* enterica sorovar Enteritidis (ATCC 13076) e *Staphylococcus aureus* (ATCC 25923). A curcumina e PVP adquiridos da Sigma-Aldrich, e o suco de laranja concentrado doado pela Louis Dreyfus (LDC). As suspensões bacterianas e de esporos foram preparadas com seus meios de crescimento específicos. As nanopartículas de curcumina com PVP foram obtidas por dispersão sólida, e caracterizadas por FTIR, MET e DSC, além da avaliação de sua citotoxicidade e capacidade antioxidante. Realizou-se a determinação da concentração inibitória mínima (CIM), bem como a concentração bactericida mínima (CBM), além da incorporação das nanopartículas no suco de laranja, com verificação da sua interferência no pH, cor e °Brix. Os resultados foram tratados por análise estatística ANOVA e teste de Tukey através do software Statistica 7.0.

RESULTADOS E DISCUSSÃO

Sobre os resultados de formação de biofilme, a menor carga microbiana levou a formação de biofilme no aço inoxidável após 48 horas de contato a 28 °C e após 24 horas a 45 °C, enquanto que na borracha a maior formação foi observada após 48 horas de contato em ambas temperaturas. A baixa carga microbiana inicial demonstrou baixa eficácia de esporulação. Com relação a maior carga microbiana foi observada formação de biofilme no aço após 4 horas de contato a 28 °C e 45 °C; e na borracha tal formação foi observada após 8 horas de contato a 28 °C e 4 horas a 45 °C. Assim, houve diferença estatística entre as temperaturas, na superfície de aço inoxidável, nas duas concentrações avaliadas; já para a superfície de borracha não houve tal diferença estatística.

Com relação aos resultados das nanopartículas de curcumina, sua eficiência foi comprovada, uma vez que as quantidades necessárias para expressar sua atividade antibacteriana foram menores que em sua forma livre, frente as bactérias deteriorantes e patogênicas testadas, como CIM de 125 μg/mL em *Staphylococcus aureus* e 62,5 μg/mL em *A. acidoterrestris*, além das análises de infravermelho por transformada de Fourier (FTIR) e calorimetria diferencial de varredura (DSC) comprovarem a eficácia da encapsulação. As imagens obtidas por microscopia eletrônica de transmissão (MET)

apresentaram uma vasta gama de tamanhos de nanopartículas. O ensaio de atividade antioxidante confirmou as propriedades bioativas da curcumina encapsulada e também sua não citotoxicidade frente a quatro linhagens de células de carcinoma e duas não tumorais. A estabilidade do suco adicionado das nanopartículas foi confirmada através da manutenção de seu pH, cor e ºBrix após 3 dias de armazenamento a 8 °C, temperatura de refrigeração.

CONCLUSÕES

Observou-se maior formação de biofilme com a maior carga microbiana inicial nas duas superfícies de contato analisadas, aço e borracha, em ambas as temperaturas, porém quando se levou em consideração o tempo de contato houve maior formação na superfície de borracha. Como a formação de biofilme já foi observada após 4 horas de contato, faz-se necessária a realização dos procedimentos de higiene frequentemente. Com relação aos esporos, estes apresentaram maior facilidade de adesão na superfície de aço inoxidável, com presença elevada a partir da maior concentração inicial de micro-organismos.

Com relação a utilização das nanopartículas de curcumina como agentes antimicrobianos, o resultado foi satisfatório, pois comprovou seu efeito antimicrobiano e antibactericida frente as cepas testadas; além da estabilidade da nanopartícula ter sido confirmada pelos ensaios realizados.

Palavras-chave: *Alicyclobacillus*, esporos, suco de laranja, biofilme, curcumina, nanopartículas.

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FOOD TECHNOLOGY

Contribution of environmental factors in the formation of biofilms by *Alicyclobacillus acidoterrestris* on surfaces of the orange juice industry

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ABSTRACT: *The objective of this study was to evaluate the effect of the initial microbial load, temperature and contact time on the biofilm formation of Alicyclobacillus acidoterrestris on stainless steel and natural food-grade rubber using orange juice as culture medium. The low initial load of A. acidoterrestris (2 log CFU/mL) led to biofilm formation on the stainless steel surface after 48 h of contact at 28 ºC and after 24 h at 45 ºC, and on natural food-grade rubber surface after 48 h of contact at both temperatures. The high initial microbial load (5 log CFU/mL) led to biofilm formation on stainless steel after 4 h of contact at 28 °C and 45 °C, while biofilm was formed on natural food-grade rubber after 8 h of contact at 28 °C and 4 h at 45 °C. The microbial load also affected the presence of spores in biofilm, which was observed on both surfaces only at high initial loads of A. acidoterrestris.*
Key words: *concentrated orange juice, stainless steel, natural food-grade rubber, spores, biofilm.*

Contribuição de fatores ambientais na formação de biofilmes por *Alicyclobacillus acidoterrestris* em superfícies da indústria de suco de laranja

RESUMO: *O objetivo deste estudo foi avaliar o efeito da carga microbiana inicial, temperatura e tempo de contato na formação de biofilme de Alicyclobacillus acidoterrestris em aço inoxidável e borracha natural de qualidade alimentar utilizando suco de laranja como meio de cultura. A baixa carga inicial de A. acidoterrestris (2 log UFC/mL) levou à formação de biofilme na superfície do aço inoxidável após 48 h de contato a 28 ºC e após 24 h a 45 ºC, e na superfície natural de borracha de qualidade alimentar após 48 h de contato nas duas temperaturas. A alta carga microbiana inicial (5 log UFC/mL) levou à formação de biofilme em aço inoxidável após 4 h de contato a 28 °C e 45 °C, enquanto o biofilme foi formado em borracha natural de qualidade alimentar após 8 h de contato a 28 °C e 4 h a 45 °C. A carga microbiana também afetou a presença de esporos no biofilme, o que foi observado em ambas as superfícies apenas com altas cargas iniciais de A. acidoterrestris.*
Palavras-chave: *suco concentrado de laranja, aço inoxidável, borracha natural de qualidade alimentar, esporos, biofilme.*

INTRODUCTION

Brazil is currently the world's leading producer and exporter of concentrated orange juice. Concentrated orange juice has low water activity (0.80 - 0.83), low pH (3.5 to 4.0), a high concentration of soluble solids (65 °Brix), high viscosity, and low redox potential, which together with the heat treatment during the concentration process inhibit the multiplication of many spoilage and pathogenic microorganisms. However, bacteria of genus *Alicyclobacillus* spp. survive these environments and caused an unpleasant taste and odour in the juice, described as antiseptic or disinfectant due to the formation of 2,4-dibromophenol and 2-methoxyphenol (guaiacol) compounds, respectively (ORR et al., 2000; SMIT et al., 2011; STEYN et al., 2011).

Alicyclobacillus is a genus of spore-forming bacteria, Gram-positive that have already been found in soil, organic compost, manure, fruit surface, and acidic beverages (STEYN et al., 2011; TIANLII et al., 2014). The contamination of juices and processing environment with *Alicyclobacillus* spp. may occur during post-harvest without adequate cleaning of the fruits. This microorganism may still be present in the food industry in the form of biofilms (ANJOS et al., 2013). Biofilms are considered a complex and structured community of microorganisms, surrounded by an extracellular matrix of polysaccharides, adhered to each other and/or to a surface or interface

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(COSTERTON et al., 1995). These biofilms increase the cell's resistance to environmental stresses, reduce the efficiency of sanitizers, and bring economic losses to the food industry, as it can be a focus of food contamination (SIMÕES et al., 2010).

The objective of this study was to evaluate the effect of the initial inoculated microbial load (low - 2 log, or high - 5 log), processing temperatures (28 °C and 45 °C) and contact times (0, 4, 8, 24, 48, and 72 h) on the biofilm formation of *A. acidoterrestris* on stainless steel and natural food-grade rubber surfaces using orange juice as culture medium.

MATERIALS AND METHODS

Materials

A. acidoterrestris CBMAI 0244T strain (DSMZ 3922, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) was used for the biofilm formation. The strain was stored in 30% glycerol at -20 °C and activated in 3 mL of BAT broth (*Bacillus acidoterrestris* broth) at 45 °C for 24 h.

The biofilm formation was evaluated in AISI 304#4 stainless steel coupon (8 mm x 8 mm x 1 mm) and a natural food-grade rubber surface (8 mm x 8 mm x 3 mm), non-toxic, food-grade rubber, normally utilized as a fruit conveyor belt in food industries. Before each assay, the surfaces were rinsed with neutral detergent and distilled water, immersed in 70% (v/v) ethanol for 1 hour at room temperature, rinsed again in distilled water, placed in microtubes, and sterilized at 121 °C for 15 (FERNANDES et al., 2014).

Concentrated orange juice (66 ºBrix) was reconstituted to 11 °Brix (aw 0.96, pH 4.0) using sterile deionized water.

Absence and control of microbial load

The absence of *Alicyclobacillus* spp. vegetative cells and spores in the samples was previously investigated. In each sterile microtube were added one coupon, 900 uL reconstituted orange juice and 100 uL diluted culture. Two experiments were carried out: i) addition of 100 uL *A. acidoterrestris* strains at a load of 2 log CFU/mL, and ii) addition of 100 uL *A. acidoterrestris* strains at 5 log CFU/mL. Subsequently, the microtubes were incubated at 28 and 45 °C. The analyses were performed after 0, 4, 8, 24, 48, and 72 h. After each inoculation, a control of *A. acidoterrestris* cell count on BAT agar was performed to confirm the initial microbial load. Plates were incubated at 45 °C for 24 h.

Biofilm formation

The biofilm formation was assessed by the plate counting technique. At each time (0, 4, 8, 24, 48, 72 h) and contact temperature (28 ºC and 45 ºC), the stainless steel and natural food-grade rubber coupons were removed from the orange juice and transferred separately to microtubes containing 1.0 mL of 0.85% saline solution, remaining immersed for 1 min at rest to remove the planktonic cells. Then, each vial was immersed in 1.0 mL of 0.85% saline solution and subjected to ultrasound for 5 min to remove the sessile cells (ANJOS et al., 2013). For the spore counts, the coupons were subsequently subjected to a heat shock of 80 °C for 10 min, followed by plating on BAT agar and incubation at 45 °C for 24 h (FERNANDES et al., 2014). The count was performed on BAT agar by drop plate method (HERIGSTAD et al., 2001). On each BAT agar plate was added three drop (20 uL each) of each dilution. The average of the counts was applied according to SWANSON et al., 1992. Each experiment was repeated three times.

Statistical analyses

All investigated variables were subjected to an analysis of variance (ANOVA). For each temperature, the contact times were compared using Tukey's test ($p<0.05$). The results of vegetative cells counts were compared between the temperatures of 28 ºC and 45 °C using T-Student test ($p<0.05$). The same test also has been used to compare results of spore counts between temperatures of 28 and 45 °C. In all cases, the statistic tests were applied separately for planktonic cells orange juice, sessile cells stainless steel, and sessile cells natural food-grade rubber, for each time. Statistical analysis was performed using the SISVAR program version 5.3 (FERREIRA, 2008).

RESULTS

Tables 1 and 2 show the results of low and high initial concentration, respectively, the *A. acidoterrestris* planktonic cells counts (log CFU/mL) in orange juice, and the sessile cells counts (log CFU/cm^2) on stainless steel and natural food-grade rubber surfaces as a function of the time and temperature.

The low initial load of *A. acidoterrestris* (Table 1) led to biofilm formation on the stainless steel surface after 48 h of contact at 28 ºC and after 24 h at 45 ºC. The highest biofilm formation ($P<0.05$) on stainless steel was observed after 72 h at 28 °C and 24 h at 45 ºC. After 72 h at 45 ºC, a reduction of the *A. acidoterrestris* counts of more than one log cycle was observed. On the natural food-grade rubber surface,

Table 1 - Mean *Alicyclobacillus acidoterrestris* count ± standard deviation (SD) of planktonic cells (log CFU/mL) and sessile cells (log CFU/cm^2) at initial inoculated load of 2 log CFU/mL.

Time (h)	----------Planktonic cells Orange juice----------				----------Sessile cells Stainless steel----------				----------Sessile cells Rubber----------			
	----------28 ºC----------		----------45 ºC----------		----------28 ºC----------		----------45 ºC----------		----------28 ºC----------		----------45 ºC----------	
	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores
4	2.64±0.01^{cA}	<1.7^{*b}	3.29±1.04^{bA}	<1.7^{b}	<3$^{** c}$	<3	<3^{c}	<3	<3^{b}	<3	<3^{b}	<3
8	2.65±0.00^{cB}	<1.7^{b}	4.20±0.00^{bA}	<1.7^{b}	<3^{c}	<3	<3^{c}	<3	<3^{b}	<3	<3^{b}	<3
24	4.17±0.19^{bA}	<1.7^{b}	4.94±1.77^{aA}	<1.7^{b}	<3^{cB}	<3	4.88±0.84^{aA}	<3	<3^{b}	<3	<3^{b}	<3
48	5.50±0.76^{aA}	2.7±0.00^{aA}	5.09±0.02^{aA}	3.00±0.00^{aA}	3.73±0.39^{bA}	<3	4.03±1.00^{a,bA}	<3	3.72±0.08^{aB}	<3	4.73±0.01^{aA}	<3
72	5.40±0.69^{aA}	2.7±0.00^{aA}	5.18±0.12^{aA}	3.48±0.00^{aA}	4.39±0.20^{aA}	<3	3.44±0.04^{bA}	<3	<3^{bB}	<3	3.98±0.47^{bA}	<3

*Detection limit = 1.7 log CFU/mLfor planktonic cells. SD not established.**Detection limit = 3 log CFU/cm^2 for sessile cells. SD not established.
^{a,b,c}Means in the same column followed by the same lowercase letter are not significantly different by the Tukey's test (P≥0.05).
^{A,B}Means in the same row followed by the same uppercase letter (comparing 28 ºC x 45 ºC for vegetative cells and comparing 28 ºC x 45 ºC for spores) are not significantly different by the T-Student test (P≥0.05). The statistic test was applied separately for planktonic cells orange juice, sessile cells stainless steel and sessile cells rubber.

the highest biofilm formation of *A. acidoterrestris* (P<0.05) occurred after 48 h of contact at 28 ºC and 45 ºC, with a reduction after 72 h, at both temperatures.

The low initial *A. acidoterrestris* population led to low sporulation efficiency of the microorganisms over time at 28 and 45 ºC (Table 1). Therefore, the presence of spores in the biofilm was not observed (count below the detection limit: <3 log CFU/cm^2).

At high initial *A. acidoterrestris* population (Table 2) led to the biofilm formation on stainless steel after 4 h of contact at both 28 °C and 45 °C. The highest biofilm formation was observed after 24 h of contact at 28 °C, although scores were not statistically different (P ≥0.05) over time. At 45 ºC after 8 h of contact the highest biofilm formation was observed (5.30 log CFU/cm^2, P<0.05). The biofilm formation was also observed on the natural food-grade rubber surface after a few hours, within 8 h and 4 h for 28 ºC and 45 ºC, respectively. On the natural food-grade rubber surface, the highest biofilm formation was observed at 28 °C after 72 h of contact, with counts of 4.56 log CFU/cm2 (P<0.05). At 45 °C the highest count was after 72 h, however, there was no significant difference with the other times of contact (P ≥0.05).

At high initial *A. acidoterrestris* population (5 log CFU/mL), the planktonic cells counts in orange juice were higher after 4 h at both 28 °C and 45 °C and over time and the biofilm formation began after a few hours of contact with both stainless steel and natural food-grade rubber surfaces (Table 2). In addition, at high initial *A. acidoterrestris* populations, the sporulation in orange juice was observed after 4 h for the two temperatures under study, thus spore formation was detected in both biofilms from stainless steel and natural food-grade rubber surfaces. However, the spore count on the stainless steel surface decreased (P<0.05) after 24 h of contact.

In the present study we verified statistical difference between the temperatures tested. For example, on the stainless steel surface at low and high concentration at 24 h and 8 h contact, respectively, the vegetative cell counts of *A. acidoterrestris* were higher at 45 ºC than at 28 °C (P<0.05). For the natural food-grade rubber surface, at high concentrations, there was no statistical difference between the evaluated temperatures (P≥0.05).

The low initial microbial load inoculated in the orange juice at 28 ºC allowed the adaptation of the bacteria with slow multiplication, thus taking more time for the biofilm formation and high microbial counts. This fact was confirmed by the planktonic cells counts in orange juice over time (Table 1). The initial (4 and 8 h) planktonic cells counts were below 3 log CFU/mL, while the high planktonic cells counts (above 4-5 log CFU/mL, P<0.05) were only observed after 48 h, precisely when the biofilm was formed. At 45 ºC, high plankton cell counts were observed after 24 h (P<0.05), when biofilm formation had already occurred.

Stainless steel surface was more propitious to biofilm of *A. acidoterrestris* formation at low

Table 2 - Mean *Alicyclobacillus acidoterrestris* counts ± SD of planktonic cells (log CFU/mL) and sessile cells (log CFU/cm^2) at initial inoculated load of 5 log CFU/mL.

Time (h)	----------Planktonic cells Orange juice----------				----------Sessile cells Stainless steel----------				----------Sessile cells Rubber----------			
	----------28 ºC----------		----------45 ºC----------		----------28 ºC----------		----------45 ºC----------		----------28 ºC----------		----------45 ºC----------	
	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores
4	5.40±1.06^{aB}	3.59±0.02^{bB}	6.35±0.08^{aA}	4.45±0.13^{aA}	3.30±0.17^{aA}	3.05±0.12^{a}	3.95±0.65^{bA}	<3^{*b}	<3^{cB}	<3^{b}	4.13±1.52^{aA}	<3^{b}
8	6.12±0.30^{aA}	4.45±0.13^{aA}	5.91±0.57^{aA}	4.38±0.05^{aA}	3.69±0.04^{aB}	3.59±1.00^{aA}	5.30±0.14^{aA2}	3.77±0.80^{aA}	3.79±0.01^{bA}	<3^{b}	4.10±0.32^{aA1}	<3^{b}
24	5.71±0.56^{aA}	4.68±0.42^{aA}	5.49±0.30^{aA}	4.58±0.20^{aA}	4.29±0.02^{aA}	<3	3.89±0.40^{bA}	<3^{b}	4.28±0.20^{a,bA}	<3^{b}	4.37±0.18^{aA}	<3^{b}
48	5.93±0.27^{aA}	5.16±0.06^{aA}	5.71±0.06^{aA}	4.25±0.05^{aB}	4.27±0.00^{aA}	<3	4.10±0.44^{a,bA}	<3^{b}	4.01±0.19^{a,bA}	3.40±0.25^{aA}	4.23±0.87^{aA}	3.53±0.48^{aA}
72	6.00±0.00^{aA}	4.61±0.03^{aA}	5.64±0.05^{aA}	3.74±0.39^{aB}	3.82±0.45^{aA}	<3	3.91±0.63^{bA}	<3^{b}	4.76±1.13^{aA}	3.61±0.73^{aA}	4.60±0.02^{aA}	3.71±0.31^{aA}

*Detection limit = 3 log CFU/cm^2 for sessile cells. SD not established.
^{a,b,c}Means in the same column followed by the same lowercase letter are not significantly different by the Tukey's test (P≥0.05).
^{A,B}Means in the same row followed by the same uppercase letter (comparing 28 ºC x 45 ºC for vegetative cells and comparing 28 ºC x 45 ºC for spores) are not significantly different by the T-Student test (P≥0.05). The statistic test was applied separately for planktonic cells orange juice, sessile cells stainless steel and sessile cells rubber.

microbial load, however, at high microbial load, after 72 h of contact, natural food-grade rubber surface was more propitious.

The high initial load (5 log CFU/mL) of *A. acidoterrestris* led to biofilm formation on the different surfaces more rapidly than low initial load (2 log CFU/mL). In this case, after 4 h of contact, biofilm formation has occurred, suggesting that hygiene procedures must be performed frequently. The microbial load can also affect the presence of spores in the biofilm formed, which was observed on both surfaces only at high initial loads of *A. acidoterrestris*.

DISCUSSION

The temperatures of 28 °C and 45 °C were selected in this study to represent the environment processing temperature and the ideal temperature of *A. acidoterrestris* growth, respectively (SMIT et al., 2011). The time interval was selected based on the equipment cleaning schedule of the orange juice industry.

Probably, the reduction in the biofilm count after 72 h on the food-grade rubber surface was due to the detachment of the biofilm cells, as the planktonic cell count in the orange juice remained high after 72 h. This is worrisome because detachment can lead to food contamination or colonization of other regions, resulting in new biofilms (SIMÕES et al., 2010).

Among the two inoculated microbial load, 5 log CFU/mL and 2 log CFU/mL, the highest biofilm formation of *A. acidoterrestris* was observed at higher microbial load, for both surfaces. Therefore, the higher the microorganism population, the greater the biofilm formation. PEÑA et al. (2014) found that the inoculation of 6 log CFU/mL of *Bacillus cereus* in milk led to a higher biofilm formation when compared with the inoculation using a low microbial population (3 log CFU/mL), demonstrating the effect of the contamination level on the biofilm formation.

Regardless of the microbial species or surface analysed, the adhesion process may occur with maximum intensity at the optimum temperature growth range (MEIRA et al., 2012). *Alicyclobacillus* spp. can grow from 20 to 70 °C, with the optimum temperature ranging from 42 to 60 °C (SMIT et al., 2011).

It is worth mentioning that the spores adhere moreeasily to the stainless steel surface, due to their hydrophobic properties (RYU & BEUCHAT, 2005), and the adhered spores become even more resistant to the cleaning procedures. Then, under favourable environmental conditions, the spores can germinate in vegetative cells and continue the multiplication process (ELHARIRY, 2011), being able to recontaminate the processed juice.

The great majority of the equipment surfaces in the juice processing industry is stainless steel, although this surface is considered smooth, it can wear away over time, with cracks and grooves and corrosion points, which also facilitate adhesion of the microorganism and subsequent biofilm formation (SIMÕES et al., 2010).

The natural food-grade rubber is a piece of the conveyor belts the fruits after the arrival at the factory. The rubber surface is usually affected by sanitizing procedures and, consequently, it wears away more easily, which favours the biofilm formation. In addition, rubber often has a porous and

spongy structure, which facilitates the adhesion of microorganisms with subsequent biofilm formation. Therefore, these characteristics of the rubber should be evaluated before its use in the food industry. To date, the literature lacks information on biofilm formation of A. acidoterrestris on rubber surfaces.

The biofilm formation of *A. acidoterrestris* in this study occurred at 28 ºC and 45 ºC. It is worth noting that both temperatures are used in the equipment during the processing of orange juice, thus the poor sanitation can contribute to the biofilm formation. Both surfaces were suitable for biofilm formation of *A. acidoterrestris*. However, over time of contact, a higher biofilm formation was observed at high microbial load on the natural food-grade rubber surface, and at low microbial load on the stainless steel surface.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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Use of nanoencapsulated curcumin against vegetative cells and spores of *Alicyclobacillus* spp. in industrialized orange juice

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ABSTRACT

Pathogenic and deteriorating bacteria are a great concern to food safety. In this sense, the present study evaluated the fight against microbial contamination through the use of nanoparticles containing curcumin, in addition to analyzing the physical properties of these nanoparticles. Efficient curcumin encapsulation was determined by Fourier transform infrared spectra evaluation and differential scanning calorimetry. Transmission electron microscopy images showed irregular shaped nanoparticles with broad size distribution (20–250 nm). The antibacterial activity was considered satisfactory, since curcumin in the form of nanoparticles demonstrated antimicrobial and antibacterial activity superior to curcumin in its free form, against both pathogenic bacteria, such as *Staphylococcus aureus* (MIC 125 μg/mL), and deteriorates, such as *Alicyclobacillus acidoterrestris* (MIC 62.5 μg/mL). Since curcumin nanoparticles may be consumed as a food additive, the bioactive properties of the nanoencapsulated curcumin were also evaluated in relation to antioxidant capacity (Thiobarbituric acid reactive substances (TBARS) and oxidative hemolysis inhibition assays) and cytotoxicity against four carcinoma cell lines, as well as two non-tumor cells. As a proof of concept, nanoparticles were incorporated in orange juice, with the juice maintaining satisfactory pH, °Brix, and color stability, during three days of storage (8 °C).

1. Introduction

The food production and consumption chains are increasingly concerned about the microbiological safety of food. There are several disinfection methods available and already implemented in the food industry; however, techniques that do not alter the organoleptic properties of foods are still lacking (Spricigo et al., 2013).

According to the World Health Organization, *Salmonella* spp. and *Staphylococcus aureus* are among the main bacteria that cause foodborne diseases (FBDs) (WHO, 2018). In addition to these, there are other significant microorganisms responsible for FBDs, such as species of *Alicyclobacillus*, which are Gram-positive and thermoacidophilic bacteria. These bacteria are capable of multiplying in a wide range of pH (2.5–6.0) and temperature (25–60 °C), and some species can even form spores as a resistance mechanism. *Alicyclobacillus* spp. are associated with the deterioration of citrus juices, concentrates, teas, and tomato extracts due to inefficient pasteurization processes (Cai et al., 2019; Wang et al., 2018). Of the 22 species that comprise this genus, *A. acidoterrestris* is considered the most important deteriorate as it can produce by-products, such as guaiacol, which causes the astringent taste and odor in juices (Chang and Kang, 2004; Ciuffreda et al., 2015; Goto et al., 2002).

Due to the characteristics of sporulation and biofilm formation, the industry seeks alternatives for the control of *Alicyclobacillus* spp. in food

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processing. The main objective, in addition to combating the microorganism, is to maintain the product's sensory characteristics. As the conventional pasteurization process is not able to eliminate *Alicyclobacillus* spp., alternatives have been investigated, including high hydrostatic pressure, ultraviolet radiation, natural antimicrobials, pulsed electric fields, ultrasound, pulsed light, or a combination of these processes with mild thermal treatments (Tremarin et al., 2019). Another promising approach that can be evaluated is the use of natural extracts with antimicrobial properties (Bevilacqua et al., 2008).

Some efforts have been made in the search for substances of natural origin with action against *Alicyclobacillus* spp., such as the use of papain and bromelain, which are proteolytic enzymes derived from papaya and pineapple, respectively (Anjos et al., 2016). Both enzymes were evaluated against strains of *A. acidoterrestris*, *A. hesperidum*, *A. acidiphilus*, *A. cycloheptanicus*, and *A. acidocaldarius*. The enzymes were reported to have effective inhibitory and bactericidal activity at low concentrations against all the strains except *A. acidocaldarius*. In another study, oregano (*Origanum vulgare*) essential oil was evaluated against the same strains, and was shown to efficiently control the growth of *Alicyclobacillus* spp. (Dutra et al., 2019). The authors highlighted the correlation between the good antioxidant activity of the compounds present in the essential oil, such as carvacrol acetate, and the antimicrobial activity of the natural product. Other natural extracts have also been studied against *Alicyclobacillus* spp., such as rosemary extract (Piskernik et al., 2016), green tea kombucha (Mizuta et al., 2020), *Piper peltatum* and *Piper marginatum* extracts (Pascoli et al., 2018), and thymol (Cai et al., 2019).

A largely studied natural antimicrobial compound is curcumin (diferuoyl methane), which is the major component within the phenolic compounds of *Curcuma longa* L. Curcumin has received considerable attention for its therapeutic properties, such as anti-inflammatory, anticancer, and antioxidant, as well as its antibacterial capacity (Gonçalves et al., 2020). Curcumin is already used in the food industry as a stabilizing agent or natural colorant (Mangolim et al., 2014; Mahmood et al., 2015), however its applicability is limited due to its poor water solubility. The encapsulation of poorly water-soluble compounds is a viable solution to overcome this problem. Generally Recognized as Safe (GRAS) materials can be used as encapsulating agents, thus increasing the range of foodstuff to which curcumin could be applied.

In view of the therapeutic potential of curcumin, Shah et al. (2018) evaluated the effects of nanoconjugates of curcumin and Ag (C-AgNPs) against skin cancer in patients. The authors reported better anti-cancer activity for the C-AgNPs conjugate ($AgNO_3$, 10–3 M, mixed with 10–5 M curcumin solution) than for the curcumin alone, suggesting its use as a chemotherapeutic agent for the treatment of cancer.

Solid dispersion is one of the encapsulation strategies that can be applied to obtain a water-soluble product from a hydrophobic compound, such as curcumin. Solid dispersions can be achieved by spray drying, melt extrusion, wet milling, and dissolution techniques. In the dissolution approach, a solvent that is able to solubilize both, the compound of interest and the encapsulating agent (polymer), is used (mainly ethanol), together with a surfactant. This mixture is submitted to a shearing process (ultrasound, rotor-stator systems, *etc.*) that increases the interaction between the encapsulating agent (carrier) and the encapsulated compound by means of hydrogen bonding (Karavas et al., 2006; Phunpee et al., 2018). The mixture may then be dried, forming a homogeneous amorphous solid solution, where the encapsulated compound and carrier are totally miscible and soluble. This approach is considered easier to apply and less expensive than other encapsulation procedures(Leimann et al., 2019).

The encapsulation of curcumin using the dissolution approach of solid dispersion was applied by H. H. S. Almeida et al., 2018; M. Almeida et al., 2018. The encapsulating material used by the authors was polyvinylpyrrolidone (PVP), while Tween 80 was used as a surfactant and sonication as means of promoting the interaction of the compounds. The antimicrobial action of the nanoparticles was investigated against *Pseudomonas aeruginosa*, *Morganella morganii*, *Klebsiella pneumoniae* ESBL (spectrum extended producer), *Klebsiella pneumoniae*, *Escherichia coli*, *Escherichia coli* ESBL, *Listeria monocytogenes*, *Enterococcus faecalis*, methicillin-resistant *Staphylococcus aureus*, and methicillin-sensitive *Staphylococcus aureus*. Results showed minimal inhibition concentration (MIC) values between 0.5 and 1 mg/mL.

With the aim of producing a safe antimicrobial agent to be applied in the food industry, and that does not alter the organoleptic and sensory properties of the product, curcumin nanoencapsulated in PVP was evaluated. Fig. 1 shows a schematic illustration of the steps evaluated. Here, the effect of curcumin was tested against *Alicyclobacillus* spp., important bacteria, being some highly food potentially spoilage species, as until now, there is no literature regarding *in natura* (unencapsulated) or nanoencapsulated curcumin against species of this genus. Furthermore, as a proof of concept, the incorporation of the nanoparticles in orange juice was evaluated in terms of the color, pH, and °Brix of the product.

2. Material and methods

2.1. Bacterial strains and reagents

The bacterial strains used were: *Alicyclobacillus acidoterrestris* DSMZ 3922^T (CBMAI 0244^T), *A. herbarius* DSMZ 13609^T (CBMAI 0246^T), *A. acidocaldarius* subsp. *rittmanni* DSMZ 11297^T (CBMAI 0245^T), *A. sendaiensis* KCTC 3843, *A. hesperidum* DSMZ 12489^T (CBMAI 0298^T), and *A. acidocaldarius* DSMZ 446^T (CBMAI 0299^T). These strains were obtained from the German Collection of Microorganisms and Cell Culture (DSZM – Deutsche Sammlung Von Mikroorganismen und Zellkulturen). Furthermore, *Salmonella enterica* serotype Enteritidis ATCC 13076 and *Staphylococcus aureus* ATCC 25923 strains were evaluated.

Curcumin (from *Curcuma longa* (Turmeric), ≥65%; Sigma-Aldrich), polyvinylpyrrolidone (PVP, average mol wt 40,000; Sigma-Aldrich), Tween 80 (Dinâmica), and absolute ethanol (P.A.; Dinâmica) were used to obtain the curcumin nanoparticles by solid dispersion. Methanol, dimethyl sulfoxide (DMSO), petroleum ether, and absolute ethanol (P.A.; Dinâmica), potassium bromide (spectroscopy grade; Sigma-Aldrich) were used in the nanoparticle analysis. Concentrated pasteurized orange juice was obtained from the company Louis Dreyfus (LDC, Paranavaí-PR, Brazil).

2.2. Preparation of bacterial suspensions

2.2.1. Bacterial suspension

The suspensions of the microorganisms, *A. acidoterrestris, A. herbarius, A. acidocaldarius, A. sendaiensis, A. hesperidum and A. acidocaldarius* subsp. *rittmanni*, were prepared by inoculating bacterial colonies from a culture plate onto BAT (*Bacillus acidoterrestris*) medium (Deinhard et al., 1987), in BAT broth and stored in an oven at 45 °C for 24 h. After the suspensions were diluted in BAT broth according to the McFarland 0.5 scale, to obtain a concentration of 1.0×10^4 μg/mL, and from the suspensions, serial dilution in 96-well plates for analysis of minimum inhibition and bactericidal concentration (MIC and MBC) was performed.

The same procedure was followed for the preparation of bacterial inoculum from *Salmonella Enteritidis* and *Staphylococcus aureus*, only replacing the agar and BAT broth with Mueller Hinton (MH) agar and broth, and stored in an oven at 35 °C for 24 h.

2.2.2. A. acidoterrestris spores

Standard spore suspensions were prepared from five colonies grown on BAT medium agar (*Bacillus acidoterrestris* medium) (Deinhard et al., 1987); that were collected with a cell culture sowing loop and transferred to tubes containing 3 mL of the same medium. The suspension was incubated at 45 °C for 24 h, then 0.3 mL was subsequently resuspended in 10 mL of BAT broth and again incubated at 45 °C for 72 h. The culture was transferred to a cryotube, centrifuged for 1 min at 10,000 rpm, followed by three washes with sterile distilled water, then stored under

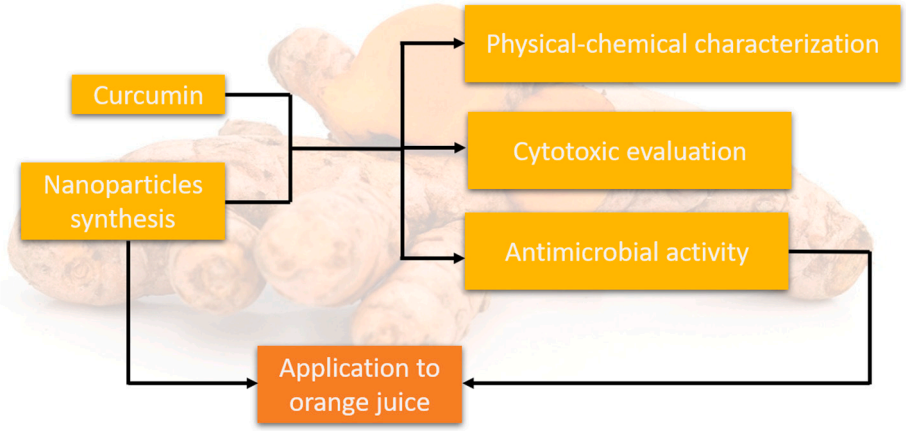

Fig. 1. Schematic illustration of curcumin nanoparticles evaluation.

refrigeration at 5 °C until use.

2.3. Curcumin nanoparticles obtained by solid dispersion

Curcumin nanoparticles were obtained by the dissolution approach of solid dispersion, according to Karavas et al. (2006) and Miranda et al. (2016) with minor modifications. PVP (100 mg) was dissolved in ethanol (27.5 mL) under gentle stirring, after which curcumin (10 mg) and tween 80 (10 mg) were added and stirring was maintained for 5 min. The mixture was then submitted to sonication (120 W and 1/8′ tip; Fisher Scientific) for 5 min under a pulse condition of 30s on and 10s off. Temperature was controlled with an ice bath. Finally, the solid dispersion was dried in a forced air oven at 40 °C for 4 h.

2.4. Nanoparticle characterization

2.4.1. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared (FTIR) spectra were acquired using a Shimadzu IRAffinity-1. Samples (PVP, *in natura* curcumin, curcumin nanoparticles, and a physical mixture of curcumin and PVP prepared in a mortar in the same proportions as used for the nanoparticles) were pelletized with potassium bromide, and spectra were collected with a resolution of 4 cm^{-1}, by combining 32 scans in the spectral range of 4000 to 400 cm^{-1}.

2.4.2. Morphological characterization

Morphological characterization of the nanoparticles was performed using transmission electron microscopy (TEM; JEOL model JEM 2100, 200 kV). The nanoparticle solid dispersion was diluted in distilled water (0.1% w/v) and dripped onto 400 mesh formvar/carbon covered copper grids. Before the analysis, grids were kept at room temperature in a desiccator with silica.

2.4.3. Thermal characterization

Thermal properties of the nanoparticles were investigated by differential scanning calorimetry (DSC; Perkin Elmer 4000). Samples (PVP, *in natura* curcumin, curcumin nanoparticles, and a physical mixture of curcumin and PVP in the same proportion as the nanoparticles) were inserted in sealed aluminum pans and analyzed under nitrogen flow (50 mL/min) and heated from 20 to 300 °C at 20 °C/min.

2.5. Antibacterial and bactericidal activity

Minimum inhibitory (MIC) and bactericidal (MBC) concentrations were determined using the 96-well microplate microdilution technique according to CLSI (2012) methodology, M7-A9. For the inoculum activation, discontinuous streaks were performed on Petri dishes containing specific growth agar, BAT agar for *Alicyclobacillus* spp. incubated at 45 °C for 24 h, and Mueller Hinton (MH) agar for *Salmonella Enteritidis* and *Staphylococcus aureus*, incubated at 35 °C for 24 h. Colonies were then isolated and cultures were pre-activated for 24 h before test by seeding in the specific media and growing at the respective temperatures for each microorganism. *In natura* curcumin was dissolved in DMSO and BAT medium, the nanoparticles were dissolved in BAT or MH medium, according to the bacteria to be tested in the assay, and these were added to the first wells of a 96-well plate at an initial concentration of 2000 μg/mL and then serially diluted. The bacterial inoculum was diluted according to the McFarland 0.5 scale (10^8 CFU), and then 5 μL of the bacterial suspension was added to each well of the 96-well plate and the plate was incubated at 45 °C for 24 h. The MIC was determined by visual analysis of turbidity. For the MBC, 10 μL of each well was plated on BAT and MH agar plates in triplicate, followed by incubation at 45 °C for 24 h, to see whether there was any subsequent growth. The same test was applied to the spores of *A. acidoterrestris*.

2.6. Cytotoxic evaluation

The cell lines of human tumors used in the cytotoxicity analysis were obtained from the Leibniz DSMZ Institute – German Collection of Microorganisms and Cell Cultures.

The evaluation of cytotoxicity was performed for the curcumin nanoparticles, as well as the PVP and curcumin alone, using the following cell lines of human tumors: gastric adenocarcinoma (AGS), breast adenocarcinoma (MCF-7), non-small cell lung carcinoma (NCI-60), and colorectal adenocarcinoma (Caco-2). Non-tumor liver primary culture (PLP2), established by our lab, and non-tumor culture from African green monkey (Vero) obtained from the ECCAC, were also evaluated. The cell lines were incubated in RPMI-1640 containing heat-inactivated fetal bovine serum (FBS; 10%), glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL), and incubated at 37 °C with humidified air and 5% CO_2.

Each cell line was prepared at 1.0×10^4 cells/well in 96-well microplates and incubated for 24 h to allow cell attachment. The compounds were added at specific concentrations (0,5 μg/mL of the concentration of 1.0×10^4 cells/well) and incubated for a further 48 h. Thereafter, cold trichloroacetic acid (10%, 100 μL) was added to fix the cells, and allowed to stand for 1 h at 4 °C. The plates were then washed 3 times with deionized water and air-dried. SRB solution (0.1% sulforhodamine B in 1% acetic acid, 100 μL) was added and the plate was incubated at room temperature for 30 min. The plates were then washed with acetic acid (1%) to remove excess SRB and allowed to air dry. Finally, adhered SRB was solubilized by the addition of Tris-HCl (10 mM, 200 μL) and the plate was read at 540 nm in a microplate reader (BioTek ELx800). For each cell line tested, the GI_{50} values,

corresponding to the concentration of extract that inhibited 50% of cell growth, was calculated. Two independent experiments were performed, each one carried out in duplicate and the results are expressed as mean values and standard deviation (SD). Ellipticine was used as a positive control (Abreu et al., 2011).

2.7. Antioxidant capacity

2.7.1. Antihemolytic activity

The antihemolytic activity of the nanoparticles, PVP, and curcumin was evaluated by the oxidative hemolysis inhibition assay (OxHLIA) described previously by Takebayashi et al. (2012) with some modifications. Sheep blood samples were collected from healthy animals and centrifuged for 5 min at 1000 *g* and 10 °C. Plasma and buffy coats were discarded and erythrocytes were first washed once with NaCl (150 mM) followed by three washes with phosphate-buffered saline (PBS; pH 7.4) (Evans et al., 2013). The erythrocyte pellet was then resuspended in PBS at 2.8% (v/v). Using a flat bottom 48-well microplate, 200 µL of erythrocyte solution was mixed with 400 µL of either PBS solution (control), sample dissolved in PBS, or water (for complete hemolysis). Trolox was used as positive control. After pre-incubation at 37 °C for 10 min with shaking, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH; 160 mM in PBS, 200 µL) was added and the optical density was measured at 690 nm (BioTek ELx800). After that, the plate was incubated under the same conditions and the optical density was measured every 10 min at the same wavelength for approximately 400 min (Takebayashi et al., 2012). The percentage of the erythrocyte population that remained intact (P) was calculated according to Eq. (1).

$$P\ (\%) = \left(\frac{S_t - CH_0}{S_0 - CH_0}\right) \times 100 \quad (1)$$

where S_t and S_0 correspond to the optical density of the sample at t and 0 min, respectively, and CH_0 is the optical density of the complete hemolysis at 0 min. The results were expressed as delayed time of hemolysis (Δt), which was calculated according to Eq. (2).

$$\Delta t\ (\text{min}) = H_{t50\ \text{Sample}} - H_{t50\ \text{Control}} \quad (2)$$

where H_{t50} is the 50% hemolytic time (min) obtained from the hemolysis curve of each antioxidant sample/control concentration. The Δt values were then correlated to the antioxidant sample concentrations (Takebayashi et al., 2012) and, from the correlation obtained, the sample concentration able to promote a Δt hemolysis delay was calculated. The results were given as IC_{50} values (µg/mL) at Δt 60, (*i.e.*, sample concentration required to keep 50% of the erythrocyte population intact for 60 min).

2.7.2. Thiobarbituric Acid Reactive Substances (TBARS) assay

For the TBARS assay, pig (*Susscrofa*) brain tissues were dissected and homogenized with Tris-HCl buffer (20 mM, pH 7.4) to obtain a homogenate (1:2 w/v). The brain tissue homogenate was then centrifuged at 3000 *g* for 10 min and the supernatant was collected. Nanoparticles, PVP, and curcumin samples (0.2 mL at different concentrations dissolved in ethanol), together with $FeSO_4$ (10 µM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL), were incubated with the brain supernatant (1:2 w/v; 0.1 mL) at 37 °C for 1 h. Then, tri-chloroacetic (28% w/v; 0.5 mL) and thiobarbituric (TBA; 2% w/v; 0.38 mL) acids were added and the mixture was heated at 80 °C for 20 min and centrifuged 3000 *g* for 5 min. The evaluation of the lipid peroxidation inhibition in porcine brain homogenates results from the reduction of TBARS by the formation of the malondialdehyde-thiobarbituric acid complex (MDA-TBA). The color intensity displayed by this complex was measured by absorbance at 532 nm (UV–Vis Specord 200 spectrophotometer, Analytik Jena, Jena, Germany). The results were expressed in values of IC_{50}, the concentration of sample necessary to obtain 50% of antioxidant activity (Santos et al., 2019).

2.8. Incorporation of nanoencapsulated curcumin in orange juice

The nanoencapsulated curcumin was incorporated in the orange juice in two concentrations, based on the MIC results for the most resistant microorganism among the *Alicyclobacillus* spp. evaluated (*A. herbarius* 0246^T with an MIC of 125 µg/mL). First, concentrated and frozen orange juice (61.5°Brix) was reconstituted with water (11.1°Brix). After that, 50 mL samples were separated into four groups in triplicate: 1) control group (reconstituted juice), 2) 1 × MIC (with nanoencapsulated curcumin added at a concentration equivalent to the MIC, which was 125 µg/mL), 3) 5 × MIC (with nanoencapsulated curcumin added at a concentration equivalent to 5-fold MIC, which was 125 × 5 = 525 µg/mL), and 4) 10 × MIC (with nanoencapsulated curcumin added at a concentration equivalent to 10-fold MIC, which was 125 × 10 = 1250 µg/mL). The samples were homogenized with a magnetic stirrer. The technological properties of color, pH, and °Brix were evaluated just after sample preparation and after 3 days of storage (common shelf life of a juice prepared at home by the reconstitution of a commercial concentrated orange juice) in the fridge (8 ± 2 °C).

For the color parameter determination, a Delta Vista 450G (Delta Color) colorimeter coupled with a liquid measurement accessory was used. A 4 mm measuring aperture was applied for the measurements of the CIELAB system parameters: L* (lightness), a* (from green (−) to red (+)), and b* (from blue (−) to yellow (+)). Also, chroma (C*) and hue angle (h°) were determined. The pH was determined with a Gehaka (PG 2000) pH meter, and the °Brix with a bench refractometer (RMT, BEL Engineering). All readings of each sample were performed in triplicate.

To evaluate the antimicrobial effect of the nanoparticle in the food matrix, *A. acidoterrestris* 0244^T was used for the subsequent assays, as it is the main spoiler of orange juice, and the best result for the *in vitro* antimicrobial activity was obtained for this strain. Using nanoparticle concentrations equivalent to the MIC for this strain, 1 × MIC (62.5 µg/mL), 4 × MIC (250 µg/mL), and 8 × MIC (500 µg/mL) were tested for their antimicrobial potential in the orange juice.

The concentrated orange juice was reconstituted with sterile water to a concentration of 11°Brix. In 24-well plates, negative control wells received only the reconstituted juice, and positive control wells received the juice and inoculum of *A. acidoterrestris* 0244^T at 5 µL/mL, as described in Section 2.5. In the other wells, different concentrations of the nanoparticles were added to the orange juice and bacteria suspension. The plate was incubated at 45 °C for 24 h. After this, 10 µL of each well was plated on BAT agar plates followed by further incubation at 45 °C for 24 h, and then counting to determine the reduction rate obtained by the use of nanoparticles.

2.9. Statistical analysis

The Student's *t*-test, analysis of variance (ANOVA), and the Tukey test at a significance level of 5% ($P < 0.05$) were used to evaluate the antioxidant capacity data in the Statistica 7.0 software (Statsoft Inc., Tulsa, OK, USA, 2004). Factorial ANOVA and Tukey's test ($P < 0.05$) were applied to color, pH, and °Brix data in Statistica 7.0 (Statsoft, USA). Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's multiple-comparison posttest. Differences between groups were considered to be significant at a *P* value of <0.05. Statistical analyses were performed with GraphPad Prism 9.0 (GraphPad Software, Inc., San Diego, CA).

3. Results and discussion

3.1. Nanoparticle characterization

Fig. 2 shows the FTIR spectra of curcumin-loaded nanoparticles, a physical mixture of PVP and curcumin, and curcumin and PVP alone.

It was possible to observe the characteristic bands of the curcumin aromatic ring at 1605 cm^{-1} (C—C) and 1508 cm^{-1} (C=C) (Lemes et al.,

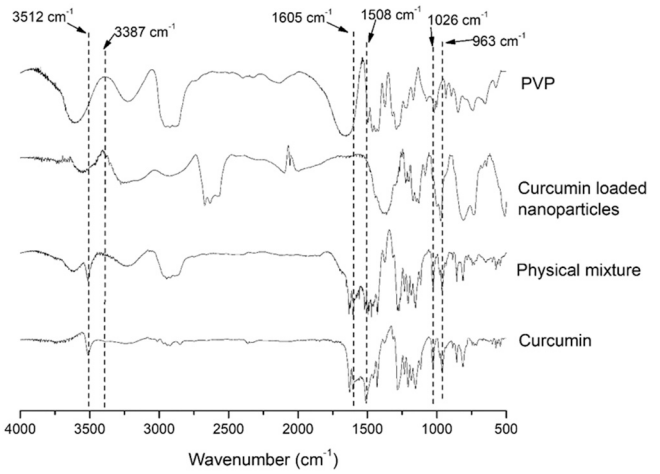

Fig. 2. FTIR spectra: PVP; to curcumin, PVP and curcumin physical mixture; curcumin loaded nanoparticles.

2017) in the free-form curcumin and the physical mixture of the compounds (curcumin and PVP), while these bands were attenuated in the nanoparticle spectra. This behavior suggests that some degree of interaction between curcumin and PVP took place in the nanoparticles, in addition to the curcumin entrapment. Furthermore, the stretching vibration of curcumin -OH can be observed at 3512 and 3387 cm^{-1} in the curcumin and physical mixture spectra; however, in the spectra of the curcumin nanoparticles spectra, these bands cannot be visualized due the presence of a bandwidth relative to adsorbed water near 3550 cm^{-1}. Also, other curcumin characteristic absorption bands can be observed at 1026 cm^{-1} (C—O groups) and 963 cm^{-1} (aromatic C—H) for the curcumin alone and the physical mixture, but these are greatly attenuated in the nanoparticle spectra, another indication of efficient encapsulation (Almeida et al., 2018a, 2018b; Silva de Sá et al., 2019).

Characterization analyses demonstrated that nanoparticles were formed (TEM images), curcumin was encapsulated properly, and the nanoparticles were stable (FTIR and DSC). In the chemical characterization analysis by FTIR, the interaction between the encapsulant bands of PVP and curcumin can be verified, as indicated by the decrease in spectrum intensity. This suggests that curcumin has been trapped efficiently, which makes the particle more soluble (Almeida et al., 2018a, 2018b). The absence of the curcumin melting temperature (Tm) for the nanoparticle samples, as determined by DSC, demonstrated that the curcumin is interacting with PVP in its amorphous form (Almeida et al., 2018a, 2018b).

The morphology of the nanoparticles can be observed in Fig. 3. Nanoparticles presented irregular shape, similar to that reported by Dong et al. (2018) who produced a solid dispersion of atorvastatin calcium with Pluronic 188, and by Almeida et al. (2018a, 2018b) who produced solid dispersions of curcumin in PVP. The size of the curcumin-loaded nanoparticles identified in the TEM images varied between 20 and 250 nm, which is in accordance with the results obtained by Almeida et al. (2018a, 2018b).

The thermal characterization of the curcumin-loaded nanoparticles is presented in Fig. 4. The melting temperature (Tm) of crystalline curcumin is clearly located at 175 °C, as observed by other authors (Lemes et al., 2017). In relation to the PVP thermogram, an endothermic peak can be seen with maximum temperature of 70 °C, which is related to the evaporation of adsorbed water, since PVP is a highly hydrophilic polymer (Almeida et al., 2018a, 2018b). In the thermogram of the physical mixture, the curcumin Tm was detected with lower intensity

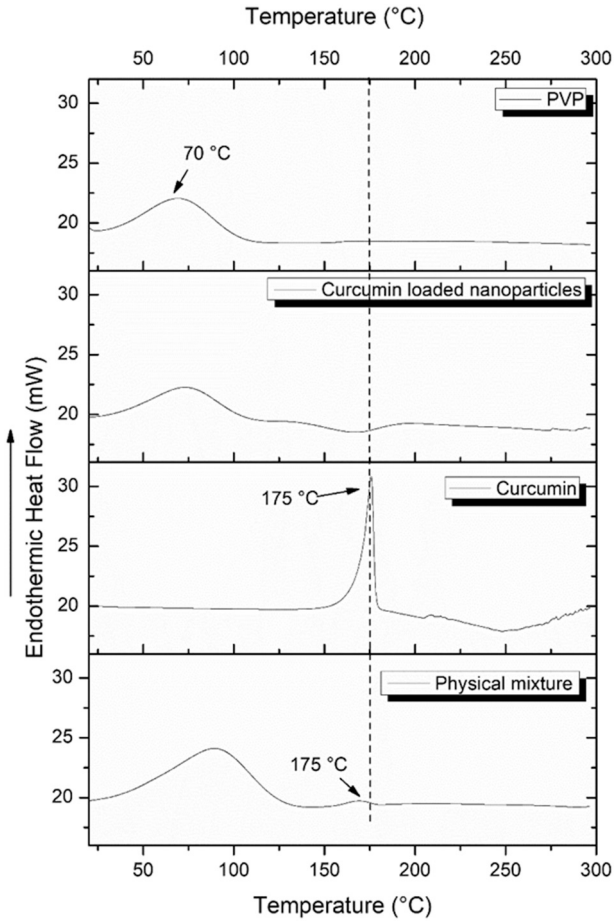

Fig. 4. DSC thermograms: PVP; to curcumin, PVP and curcumin physical mixture; curcumin loaded nanoparticles.

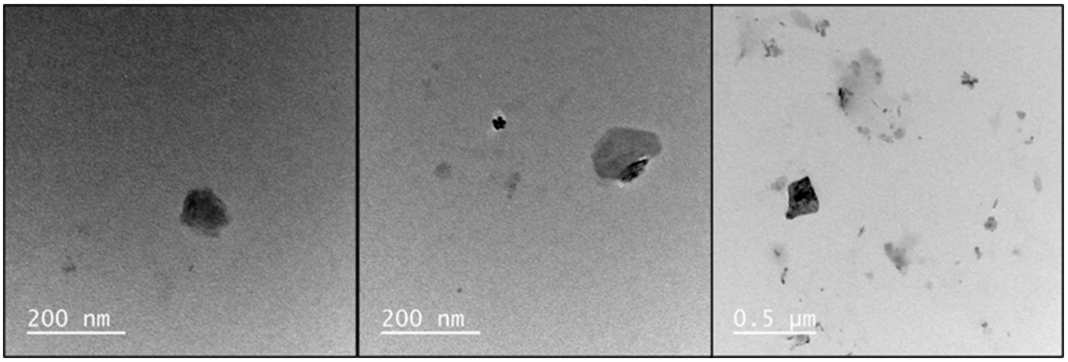

Fig. 3. Transmission electron microscopy of curcumin loaded nanoparticles.

than observed for the curcumin alone due to its proportion in relation to PVP (10% w/w) in the mixture. In the curcumin-loaded nanoparticle thermogram, the curcumin Tm was not detected, which may be considered an indication that curcumin is in its amorphous form interacting with PVP (Silva de Sá et al., 2019).

3.2. Antimicrobial activity

The MIC and MBC results for encapsulated curcumin and *in natura* curcumin against the tested microorganisms are given in Table 1. Results indicated that the *in natura* curcumin had poor activity against the microorganisms when compared to the nanoencapsulated curcumin. Typically, encapsulated curcumin presents advantages over its free form (non-encapsulated, or *in natura*), particularly regarding water solubility and bioavailability (Silva et al., 2018). Also, these nanoparticles are readily dispersible in water which favors the activity of the encapsulated compound (Almeida et al., 2018a, 2018b). Since *in natura* curcumin has low solubility in water, the compounds responsible for its antimicrobial activity are not freely available when in contact with the contaminated environment. However, in its nanoencapsulated form, with increased solubility, the compounds are released and ready to combat the microorganisms present, as observed in this study with greater antimicrobial activity observed for the curcumin in its nanoparticle form. The use of the nanoparticle technique, as demonstrated in our studies, can enhance the activity of novel antibacterial compounds with low solubility.

The sporicidal activity of curcumin nanoparticles against the spores of *A. acidoterrestris* was also evaluated. The encapsulated curcumin also presented superior action (62.5 μg/mL) against the spores than the *in natura* curcumin (1000 μg/mL), further proving the encapsulation efficiency of curcumin by the solid dispersion.

Lyu et al. (2020) evaluated the antimicrobial efficiency of silver nanoparticles (Ag) combined with curcumin (C) in a complex with oxidized amylose (AO), using *S. aureus* as an example of a Gram-positive bacteria. The authors obtained similar results to the present work, where the AO-Ag-C together showed greater antimicrobial capacity when compared to the oxidized amylose with silver alone (AO-Ag) or with curcumin alone (AO-C). The concentration determined by the researchers was equal to 2.5 mg/mL of the AO-Ag-C solution, higher than the result found in this work (Table 1).

Mirzahosseinipour et al. (2020) evaluated, through antimicrobial photodynamic therapy, the action of nanoparticles of curcumin and silica delivered to planktonic cells of the Gram-positive *S. aureus* and the Gram-negative *P. aeruginosa*. The authors obtained a reduction of 1.2 log CFU/mL in the count of *S. aureus* cells, and 1 log CFU/mL for *P. aeruginosa*, when using a concentration of 1 mg/mL of the curcumin-silica nanoparticles. The Gram-positive bacteria demonstrated a greater susceptibility to these nanoparticles in LED light, probably due to the difference in their cell wall structure. Gram-positive cells have a cytoplasmic membrane covered by a simple cell wall, which would facilitate the internalization of photosensitizers. However, in the present study a more significant reduction, equal to 4 log CFU/mL, was obtained with the curcumin-loaded nanoparticle (at a concentration of 125 μg/mL) against *S. aureus*, with a smaller reduction against the Gram-positive *Alicyclobacillus* strains tested.

Rai et al. (2008) identified the mechanism of antimicrobial action of curcumin as targeting FtsZ, a prokaryotic homologue of the eukaryotic cytoskeleton protein, tubulin. FtsZ is responsible for forming the Z ring in the intermediate cell that leads to bacterial division and multiplication. Curcumin in contact with FtsZ leads to a disturbance in the formation of this ring that inhibits bacterial cytokinesis. Strong inhibition of cytokinetic Z ring formation by curcumin was observed in *Bacillus subtilis* 168. The curcumin bound to FtsZ *in vitro* with a dissociation constant of 7.3 ± 1.8 μM, in addition to increasing GTPase. Therefore, the authors concluded that by disturbing the GTPase activity of FtsZ ring assembly, curcumin is lethal to bacteria, inhibiting cell proliferation (Figure Supplementary Fig. 1).

Table 1
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determined for curcumin-loaded nanoparticles and *in natura* curcumin.

	Curcumin-loaded nanoparticles		*In natura* curcumin	
	MIC (μg/mL)	MBC (μg/mL)	MIC (μg/mL)	MBC (μg/mL)
A. acdioterrestris 0244^T	62.5 ± 0.00	500 ± 0.00	1000 ± 0.00	>1000 ± 0.00
A. herbarius 0246^T	125 ± 0.00	250 ± 0.00	>1000 ± 0.00	>1000 ± 0.00
A. acidocaldarius subsp. *rittmanni* 0245^T	62.5 ± 0.00	125 ± 0.00	>1000 ± 0.00	>1000 ± 0.00
A. sendaiensis KCTC 3843	125 ± 0.00	250 ± 0.00	>1000 ± 0.00	>1000 ± 0.00
A. hesperidum 0298^T	62.5 ± 0.00	125 ± 0.00	>1000 ± 0.00	>1000 ± 0.00
A. acidocaldarius 0299^T	125 ± 0.00	250 ± 0.00	1000 ± 0.00	1000 ± 0.00
Salmonella Enteritidis ATCC 13076	1000 ± 0.00	>1000 ± 0.00	>1000 ± 0.00	>1000 ± 0.00
Staphylococcus aureus ATCC 25923	125 ± 0.00	1000 ± 0.00	1000 ± 0.00	>1000 ± 0.00

Results expressed as mean ± standard deviation.

3.3. Cytotoxicity in cell lines

The results obtained from the cytotoxicity assay with cell lines are presented in Fig. 5.

Nanoencapsulated curcumin showed increased anti-tumoral potential against the four cell lines evaluated, when compared to free form curcumin. The most effective nanoparticle action was against the gastric adenocarcinoma (AGS), since the GI_{50} concentration determined was 4.1-fold higher than curcumin in its free form. Nanoparticles presented the following importance against the tumor cell lines: AGS > MCF7 > NCI-60 > Caco-2. It is worth noting that the encapsulating agent (PVP) presented low cytotoxicity against all the cell lines, including the non-tumor PLP-2 and Vero cells. Furthermore, encapsulation modulated the cytotoxicity of curcumin against PLP-2 and Vero cells, reducing its cytotoxic effect. Santos et al. (2019) also found that with the curcuminoids (curcumin, demethoxy curcumin, and bisdemethoxycurcumin) encapsulation in PVP there was a decrease in the cytotoxicity against PLP-2 cells. Also, in relation to PLP-2 cells, Almeida et al. (2018a, 2018b) found the same pattern between unencapsulated curcumin and the curcumin encapsulated in PVP under the same conditions applied in the present work. According to Chankhampan et al. (2014), the use of biocompatible polymers in the encapsulation process allows a better toleration by the cells. Still, according to González et al. (2019), these compounds avoid normal tissues and accumulate only in tumors, due to the action of nanocarriers. In work presented by Niza et al. (2019), doxorubicin was encapsulated in devices based on tailored bare polycaprolactone with the intent of acting on glioblastoma. The authors evaluated the cytotoxicity against the tumor cell lines (C6, U87, A2780S, and A2780R), as well as against non-tumor cell lines (astrocytes and murine macrophages as these are immune cells present in the surrounding tissue of the tumor). Results showed that the treatment of non-tumor cells with free doxorubicin induced a drastic reduction of mitochondrial function that was significantly lower when these cells were treated with nanoencapsulated doxorubicin. This suggested that there was sustained released of the drug from the nanocarrier, which allowed the cytotoxic effect on the tumor cells whilst reducing the cytotoxic effect in the surrounding healthy tissue.

3.4. Antioxidant capacity

The antioxidant capacity of the nanoencapsulated curcumin, in

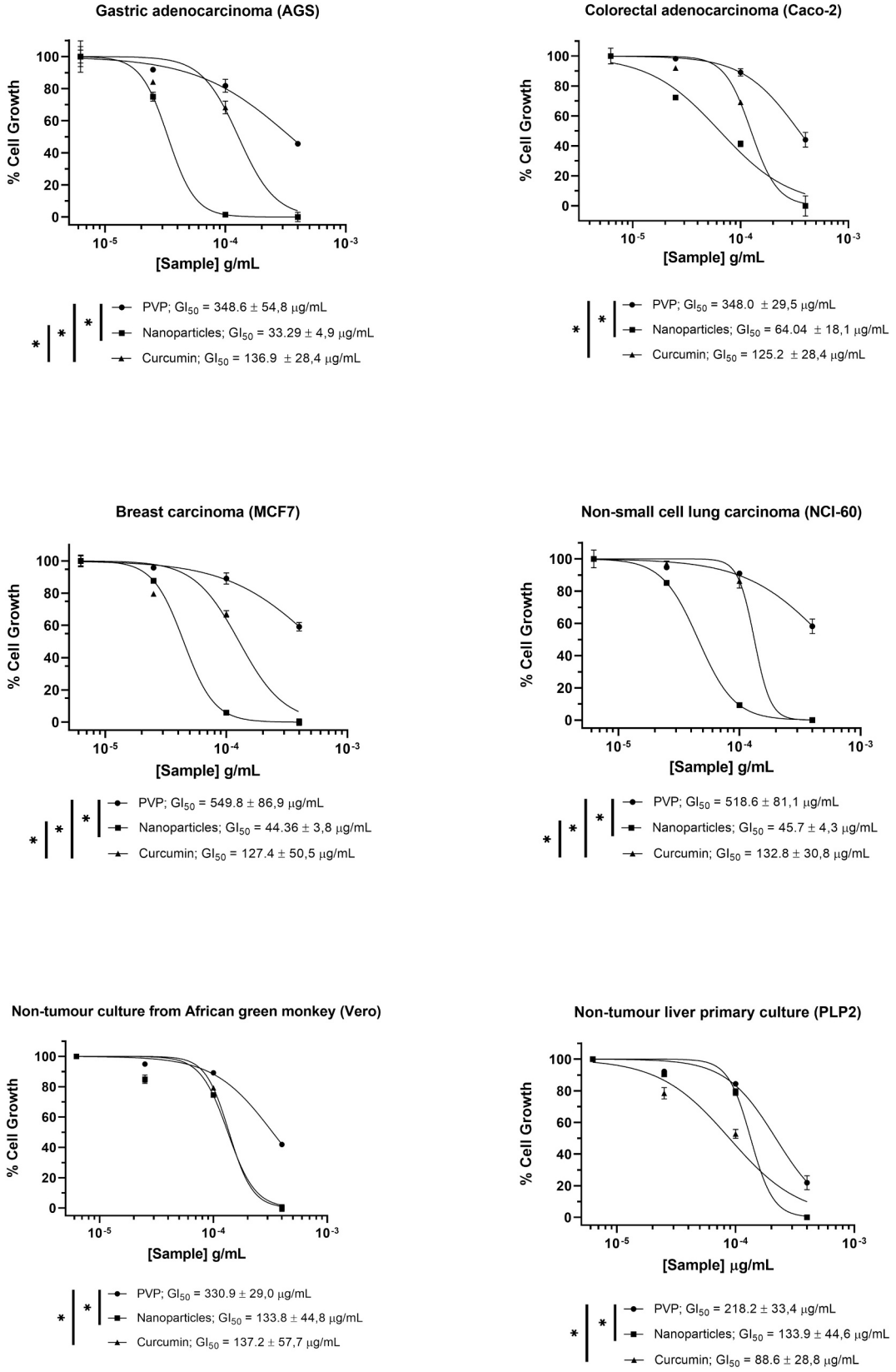

Fig. 5. Cytotoxicity assay results obtained for nanoencapsulated curcumin, the encapsulating agent PVP and curcumin in its free form (Bars with * mean $P < 0.05$ between the samples).

terms of lipid peroxidation inhibition (TBARS) and antihemolytic activity, is presented in Table 2.

The TBARS assay provides information on the compound's capacity to inhibit the formation of thiobarbituric acid reactive substances, such as malondialdehyde generated from the *ex vivo* decomposition of lipid peroxidation products. It is possible to observe in Table 2 that free form curcumin had higher antioxidant capacity when compared to the nanoencapsulated curcumin ($P < 0.05$). The same behavior was identified by Santos et al. (2019), as the authors found an IG_{50} 11.4-fold higher for curcuminoids when compared to the encapsulated curcuminoids. In the present work there was a significant difference detected ($P < 0.05$), however this difference was only 1.2-fold higher.

In relation to the oxidative hemolysis inhibition assay (OxHLIA), the IC_{50} values (μg/mL) at Δt 60 min were determined, that is, the concentration required to protect 50% of the erythrocyte population (P) from the hemolytic action caused by the used oxidizing agent for 60 min. Peroxyl radicals generated from 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) attack the biomembranes of erythrocytes and eventually cause hemolysis, that can be inhibited by antioxidant activities. This test presents many advantages over DPPH and $ORAC_{FL}$, especially as the results obtained reflect biologically relevant radical-scavenging activity (Takebayashi et al., 2012). Results presented in Table 2 show that the action of nanoencapsulated curcumin is statistical the same of free form curcumin at Δt 60 min ($P > 0.05$). On the other hand, the encapsulating agent (PVP) did not show expressive antioxidant capacity when compared to the nanoparticles, curcumin, and Trolox. It is worth noting that to make the curcumin evaluation possible (for both TBARS and OxHLIA), it was first dissolved in DMSO due to its poor water solubility. In this sense nanoencapsulated curcumin reaches the goal of bioavailability with efficient water dispersion and action in simulated biological systems.

In their review, Kunnumakkara et al. (2019) described the extensive therapeutic potential of curcumin through several clinical trials, combating chronic diseases such as cardiovascular disease, inflammatory disease, metabolic disease, neurological disease, skin disease, liver disease, and various types of cancer. It was suggested that oral or topical curcumin was mostly well tolerated. Through the association of curcumin with other compounds or formulations, such as in the form of nanoparticles, micelles, liposomes, phospholipids, and exosomes, the bioavailability of this compound is greatly enhanced. In another review, Nair et al. (2019) analyzed the anti-cancer effects of the non-curcuminoid compounds present in the curcuma rhizome in various formulations and noted that their effect can be complementary to that of curcuminoids, thus enabling the joint use of these compounds in natural cancer treatments.

3.5. Technological properties of nanoencapsulated curcumin in orange juice

Bacteria of the genus *Alicyclobacillus* can affect the quality of industrially processed juices (Prado et al., 2019). Since curcumin nanoparticles presented promising results against these bacteria, the technological properties of juice were evaluated following the incorporation of the nanoparticles in order to determine the feasibility of their use in the food industry.

Table 2
Antioxidant capacity evaluation of encapsulated curcumin (nanoparticles), the encapsulating agent (PVP) and *in natura* curcumin.

	TBARS (IC_{50}; μg/mL)	OxHLIA (IC_{50}; μg/mL) Δt = 60 min
PVP	Nd*	$1838.5^{c} \pm 27.2$
Nanoparticles	$78.0^{c} \pm 2.9$	$84.0^{b} \pm 3.0$
Curcumin	$63.0^{b} \pm 2.7$	$99.0^{b} \pm 2.0$
Trolox	$5.4^{a} \pm 0.2$	$21.8^{a} \pm 0.2$

Nd*- not detected; Results expressed as mean ± standard deviation; ^{a,b,c}different letters in the same column indicate significant difference between the treatments by Tukey's test ($P < 0.05$).

Color parameters and pH of the orange juice prepared with nanoencapsulated curcumin are presented in Fig. 6. The MIC applied in the tests was equal to 125 μg/mL, since *A. herbarius* 0246^{T} was the most resistant microorganism, among the *Alicyclobacillus* strains tested (Table 1). The addition of nanoparticles, as well as the storage time, did not affect significantly affect the °Brix results.

There was a significant difference ($P < 0.05$) in the pH, mainly associated with the storage time, but not between the orange juice with and without the nanoparticles. This result is likely due to the acid hydrolysis of polysaccharides into monosaccharides and disaccharides (Singh and Sharma, 2017). Similar results were obtained by Porto et al. (2017) and Azadbakht et al. (2021).

The addition of nanoparticles significantly affected all the color parameters. A significant decrease in luminosity (L*) occurred when nanoparticles were added at the 10 and 5-fold MIC concentrations, when compared to the control. After 3 days of storage, the control samples did not show L* variation when compared to the initial day of evaluation. All the other samples presented significantly lower L* values. On the other hand, a significant increase in the a* parameter was detected (redness tendency for positive a* values) when nanoparticles were added, which was expected since the nanoparticles have an orange-yellow color. Also, a significant increase in the b* parameter was found when the nanoparticle concentration was increased. Storage time affected the a* and b* parameters of all the samples, with the exception of the control and the 5-fold MIC samples that remained constant over the three days.

Hue angle (°h) and chroma (C*) that represent color classification (red, yellow, blue, *etc.*) and saturation or intensity respectively, are derived from a* and b*. These parameters may accurately describe color measurements and are more effective for the judgement of color analysis (McGuire, 1992). The °h scale indicates a yellowish color for values in the range of 90°, and reddish color in the range of 0° (Nanda et al., 2020). In Fig. 6 it is possible to observe that control samples tended to be yellow while nanoparticles added to the orange juice had lower °h values, associated with an orange color (yellow and red mixtures). Higher C* values were obtained for orange juice samples with the nanoencapsulated curcumin, thus showing a more saturated color when compared to the control. Color saturation was statistically the same during the storage time for the 5-fold MIC sample. According to Lee et al. (2013), the food color with a higher chroma value is preferred by consumers, moreover, in the case of yellowish foods, a higher hue angle is also preferred. The orange juice samples with nanoparticles added at the 5-fold MIC concentration should be submitted to a sensory analysis test to confirm this hypothesis, since it showed high values of both hue angle and chroma, as well as good color stability during the storage time.

The results obtained for the antimicrobial test of the nanoparticles in reconstituted orange juice are shown in Fig. 7; these showed a reduction in the microbial concentration with the different nanoparticle concentrations applied, confirming the results obtained *in vitro*. The positive control showed a growth of 6.31 log CFU/mL of *A. acidoterrestris* 0244^{T}, after 24 h, while the growth following application of 1 × MIC of the nanoparticles (62.5 μg/mL) was 4.52 log CFU/mL, for 4 × MIC (250 μg/mL) the growth was 2.46 log CFU/mL, and for 8 × MIC (500 μg/mL) the growth was 1.04 log CFU/mL. Thus, the application of nanoparticles at a concentration of 8 × MIC reduced *A. acidoterrestris* contamination in orange juice by more than 5 logs CFU/mL, indicating its possible use as an antimicrobial agent in citrus beverage industries.

4. Conclusion

Curcumin encapsulated in polyvinylpyrrolidone (PVP) nanoparticles showed antimicrobial and antibacterial activities against strains of *Alicyclobacillus* spp., as well as against the pathogenic bacteria, *S. aureus* and *Salmonella Enteritidis*, and the spores of *A. acidoterrestris*. The

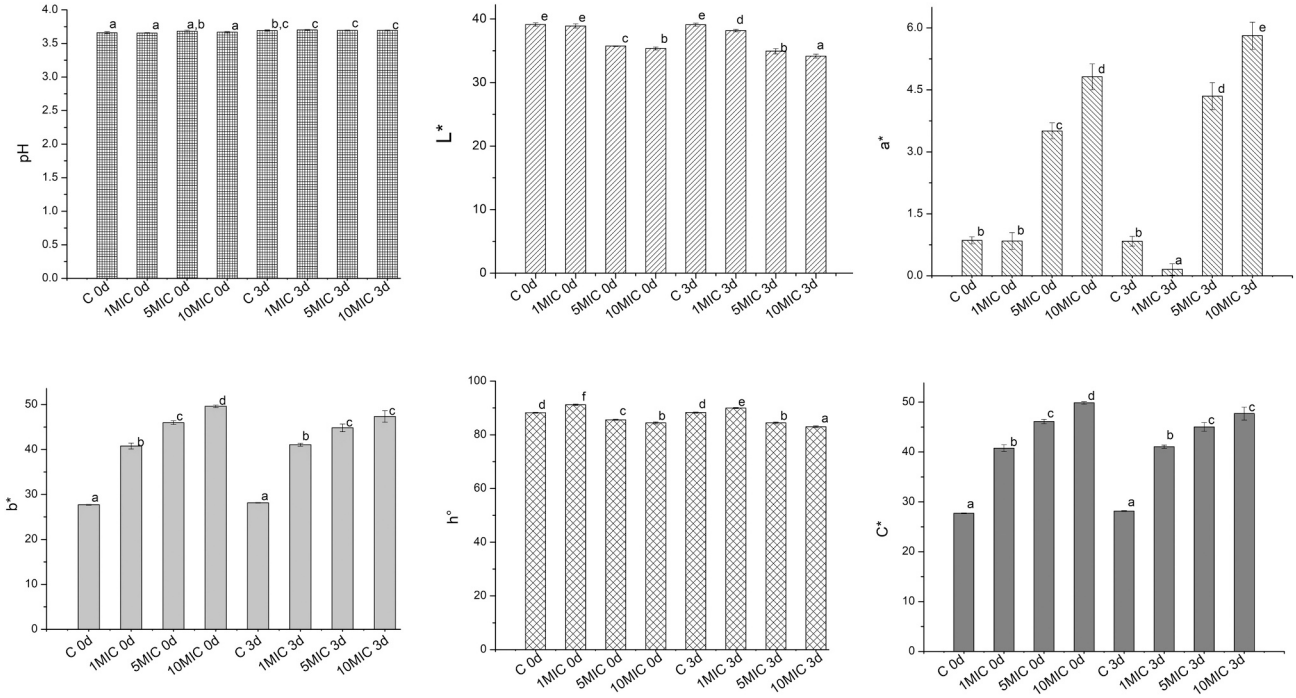

Fig. 6. Technological properties (pH, color parameters: L* (luminosity), a* (from green (−) to red (+)), and b* (from blue (−) to yellow (+), h° (hue angle) and C* (chroma)) of the orange juice added with nanoencapsulated curcumin: control, 1MIC 0 d (1-fold MIC, 0 day of storage); 5MIC 0 d (5-fold MIC, 0 day of storage), 10MIC 0 d (10-fold MIC, 0 day of storage), 1MIC 3 d (1-fold MIC, 3 days of storage), 5MIC 3 d (5-fold MIC, 3 days of storage), 10MIC 3 d (10-fold MIC, 3 days of storage). MIC = 125 μg/mL. a,b Averages followed by different letters presents significant difference by Tukey's test ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

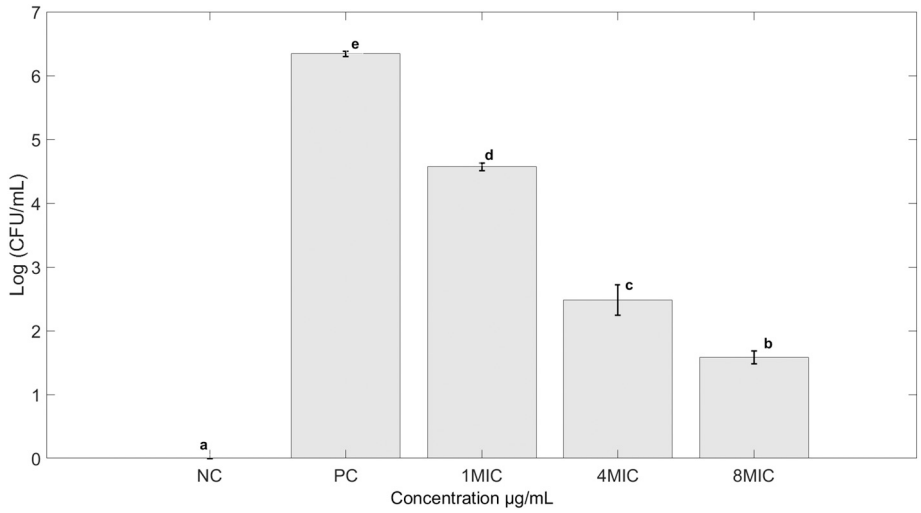

Fig. 7. Antimicrobial activity of nanoparticles against *A. acidoterrestris* 0244^T in reconstituted orange juice for 24 h at 45 °C. Concentrations of the curcumin nanoparticles in MIC values, wherein 1MIC is equal to 62.5 μg/mL, 4MIC is equal to 250 μg/mL and 8MIC is equal to 1000 μg/mL.
*NC: negative control; PC: positive control. Averages followed by different letters presents significant difference by Anova and Tukey's test ($P < 0.05$).

thermal characterization of the nanoparticles by DSC demonstrated that curcumin is in its amorphous phase, due to its interaction with PVP, as corroborated by FTIR spectra. TEM images demonstrated a large size distribution of the nanoparticles. The cytotoxicity of encapsulated curcumin against non-tumor cell lines was reduced, when compared to *in natura* (free form) curcumin. These findings demonstrate the stability of the nanoparticles. This study highlights the importance of choosing an encapsulating agent that potentiates the effects of the compound to be studied, in this case curcumin with PVP. This nanoparticle combination resulted in an improvement in the antimicrobial capacity, as well as a reduction in toxicity, providing an alternative natural product to be used by the food industry to combat microbial contamination. The produced nanoparticles were incorporated into orange juice samples, under two concentrations, as defined by the MIC of the most resistant *Alicyclobacillus* strain. When applied at the 5-fold MIC concentration, good pH, °Brix, and color stability were determined during three days of storage (8 °C), which should be studied further in terms of sensory preference.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2021.109442.

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