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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

Tatiane Viana Dutra[1] Daniela Biral do Prado[2] Márcia Maria dos Anjos[1] Miguel Machinski Junior[4] Jane Martha Graton Mikcha[3] Benício Alves de Abreu Filho[4*]

[1]Programa de Pós-graduação em Ciência de Alimentos, Universidade Estadual de Maringá (UEM), Maringá, PR, Brasil.
[2]Docente do curso de Nutrição do Centro Universitário Ingá (UNINGÁ), Maringá, PR, Brasil.
[3]Departamento de Análises Clínicas e Biomedicina Universidade Estadual de Maringá (UEM), Maringá, PR, Brasil.
[4]Departamento de Ciências Básicas da Saúde, Universidade Estadual de Maringá (UEM), 87020-900, Maringá, PR, Brasil. E-mail: baafilho@uem.br. *Corresponding author.

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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