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Alicyclobacillus acidoterrestris: Resistência a sanitizantes industriais, multiplicação e produção de biofilme

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BIOGRAFIA

Márcia Maria dos Anjos nasceu em 20 de novembro de 1985 na cidade de Ponta Grossa, Paraná. Possui graduação em Tecnologia de Alimentos pela Universidade Tecnológica Federal do Paraná. Possui especialização em Higiene e Inspeção de Produtos de Origem Animal pelo Centro Universitário de Maringá. Tem experiência nas áreas de Microbiologia e Controle de Qualidade, atuando principalmente nos seguintes temas: Controle de Qualidade de Bovinos e Laticínios, Análises Microbiológicas de Alimentos, Microbiologia Industrial, Bactérias termoacidorresistentes em suco de laranja.

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APRESENTAÇÃO

Esta dissertação de mestrado está apresentada na forma de dois artigos científicos.

1 Márcia Maria dos Anjos; Suelen Pereira Ruiz; Benício Alves de Abreu Filho. **Evaluation of *Alicyclobacillus* spp. growth enriched in orange juice in different culture media.** Revista do Instituto Biológico de São Paulo. Em avaliação.

2 Márcia Maria dos Anjos, Suelen Pereira Ruiz, Celso Vataru Nakamura, Benício Alves de Abreu Filho. **The resistance of *Alicyclobacillus acidoterrestris* spores and biofilm to industrial sanitisers.** Journal of Food Protection. Artigo aceito.

GENERAL ABSTRACT

INTRODUCTION. Genus *Alicyclobacillus* spp. is composed of gram positive bacilli with cyclic fatty acids as the main component of the cell membrane. The forming spores are the main factors of resistance to the thermal processes in food. Growth pH ranges between 2.2 and 6.0 and temperature lies between 35ºC and 55ºC. Bacteria may change the sensorial characteristics of food, such as citric juices, since they produce guaiacol (2-methoxyphenol) providing unpleasant smell and taste to the products. These microorganisms are often associated to the degradation of reconstituted orange juice. In fact, it is unavoidable that fruits bear the bacterium or its spores to the respective industrial processes since the genus is widely distributed in the soil. Orange juice is the only Brazilian product that makes up 50% of world production and 85% of Brazilian exports. Consequently, it contributes significantly towards the country´s commercial balance; its deterioration brings high liabilities. Due to different pH bands and best temperatures for the growth of different species, it is currently difficult to identify a standard methodology for the development of *Alicyclobacillus* spp. In the case of bacteria control in the industrial juice processing, the industry employs procedures, such as fruit washing, aspersion by sanitizing products and pasteurization of the product. However, spores and biofilm resist all these procedures and the bacterium may nevertheless be present in the final product. Certain factors, such as concentration and contact time between sanitizing products and the microorganism, reduce the bacteria, but no conclusive data are extant on the activities of the sanitation products used in fruit washing on the spores and on the biofilm of *Alicyclobacillus* spp.

AIMS. Current experiment evaluates the recovery and growth of three species of *Alicyclobacillus (A. acidoterrestris, A. acidocaldarius* and *A. pomorum-like*) in the media currently employed in the literature: ALI agar (*Alicyclobacillus* medium), BAT (*Bacillus acidoterrestris* thermophilic agar), K-agar and YSG agar (Yeast extract starch glucose); evaluates the adherence and the formation of the biofilm of *Alicyclobacillus acidoterrestris* on the surfaces of the industrial processing of orange juice (stainless steel, PVC – polyvynil polychloride – nylon); evaluates the efficiency of sanitizing products such as peracetic acid, calcium hypochloride and quaternary ammonium in the removal of the biofilm and in the inactivation of the microorganism´s spores.

MATERIALS AND METHODS. Two strains (*A. acidoterrestris* DSM 3922^T e *A. acidocaldarius* DSM 446^T) were obtained from the German Collection of Microorganisms and Cell Cultures (DSZM – Deutsche Sammlung Von Mikroorganismen und Zellkulturen). Three species of *Alicyclobacillus* from the concentrated orange juice industries, identified and stored in the Brazilian Collection of Microorganisms of Environment and Industry (CBMAI, Campinas SP Brazil) were employed: *A. acidoterrestris* – CBMAI 0281; *A. acidocaldarius* – CBMAI 0294 e *A. pomorum-like* – CBMAI 0278. Culture media were prepared following legal protocols found in the literature, with pH for all media standardized to 4.0. Tested media were agar (*Alicyclobacillus* medium), BAT (*Bacillus acidoterrestris* thermophilic agar), K-agar and YSG agar (Yeast extract starch glucose). Tested surfaces for the production of biofilm were stainless steel coupons AISI 304 (1.0 x 1.0 x 0.1 cm), PVC

– polyvynil polychloride (1.0 × 1.0 × 0.1 cm) and nylon bristles (1.0 × 0.1 X 0.1 cm), with surfaces washed one by one, hygienized and sterilized. Three chemical agents were selected: peracetic acid, sodium hypochloride and quaternary ammonium. Numbering of reference strains and isolates in the four media selected was undertaken by surface plating and incubation at 45ºC for 5 days. The numbering of *Alicyclobacillus acidoterrestris* with previous enrichment in reconstituted orange juice was also performed. *A. acidoterrestris* was inoculated in tubes with different surfaces to verify cell adherence to the surface. Treatment with sanitizing agents was them conducted. Quantification was undertaken by detachment of cells by sonication and plating. Adherence and sanitization were also assessed by scanning microscopy. Sporicide concentration for each sanitizing agent was also determined by microdilution. Flow cytometry with propidium iodide was performed to see whether sanitizing action affected the bacterium´s cell membrane. All assays were done in triplicate. Data were analyzed statistically by Assistat 7.6 Beta, while $p<0.05$ indicated significant difference.

RESULTS AND DISCUSSION. Plating in medium K agar in growth assays in culture media showed a low recovery of microorganisms when compared to the other three culture media. In the case of recovery of *A. acidocaldarius* and *A. pomorum-like* strains, significant differences existed for medium K agar when compared to other media but there was no significant difference between colony counts in different culture media for assays with *A. acidoterrestris* at 0h and after 24h of enrichment in juice at 45 °C. There was no significant difference between plating methods (surface and depth) employed in the assays. Characteristics in colonies of media ALI, BAT and YSG were similar, featuring circular, opaque colonies with regular light cream color margins, although YSG exhibited some slightly translucent colonies. The colonies in K agar medium had different morphological characteristics from other culture media, featuring colonies with irregular margins and some translucent colonies. In the case of adherence assays and biofilm formation, the biofilm in steel and nylon surfaces had a great cell adherence than that for PVC in all experimental conditions. Scanning electron microscopy showed the formation of a biofilm on the steel and nylon surfaces. PVC surface exhibited the adherence of cells and the production of extrapolysaccharides, although counts failed to supersede 5.0 Log UFC/cm^2 after five days of incubation. When treatment with sanitizing agents on the surfaces after cell adherence is taken into account, all treatments showed significant differences from control, except treatment with 1000 ppm sodium hypochloride in PVC and nylon surfaces which failed to show any significant decrease of adhered cells. Moreover, 1000 ppm peracetic acid treatment had the highest efficiency since it reduced more than 2 Log UFC/cm^2 in all surfaces. In the case of assays with spores, sanitizing agent with the highest sporicide activity was quaternary ammonium. Concentration 40.35 ppm completely inactivated the spores after a 10 min contact. Ammonium quaternary inactivated the spores and vegetative cells at low concentrations, although the latter were not efficient in the inactivation of *A. acidoterrestris* biofilm. Flow cytometry graphs suggest that the principal mechanism of sanitizing agents in the vegetative cell and in the spores of *A. acidoterrestris* do not occur because of membrane rupture since propidium iodide has a low fixation in the microorganism structures after treatment with sanitizing agents.

CONCLUSIONS. Comparisons between media ALI, BAT, K agar and YSG showed that media ALI, BAT and YSG recovered the initial population of different inoculums

with no significant differences among results. Advantage lies in the easiness to prepare media ALI and YSG when compared to that of medium BAT. Medium K agar had a lower recovery for all inoculums, with significant differences for the recovery of *Alicyclobacillus acidocaldarius* CMAI 0298^T and *Alicyclobacillus pomorum-like* CBMAI 0278. This fact shows that medium at pH 4.0 is not the best for the recovery of alicyclobacilli in orange juices with surface plating and incubation at 45°C for 72 to 120 h. Based on the results on cell amounts and scanning electron microscopy, it may be verified that adherence and formation of biofilm by *A. acidoterrestris* on the surfaces of stainless steel, PVC and nylon surfaces occurred. Sanitizing agents tested were not efficient in the total removal of biofilm cells of *A. acidoterrestris* for the evaluated conditions. Peracetic acid was the sanitizing agent with the highest reduction in cell numbers. In the case of all tested sanitizing agents, the double concentration, a bactericide for vegetative cells, only decreased approximately 2 Log UFC/cm^2 after adherence and biofilm formation on the surfaces. The best sporicide activity occurred with quaternary ammonium which at low concentrations and contact time inactivated spores. Sanitization by peracetic acid and quaternary ammonium are highly efficient against *Alicyclobacillus acidoterrestris* for industries. However, they are not likely to be applied in the same hygienization process due to costs and practicability. For better results, recommended concentrations of sanitizing products used by the industry should be based on stricter conditions, taking into account the specific resistance mechanisms of each bacterium and of biofilm and spore formation.

Keywords: *Alicyclobacillus*, culture medium, spores, sanitizing agent, biofilm, orange juice.

RESUMO GERAL

INTRODUÇÃO. O gênero *Alicyclobacillus* spp. é composto por bacilos Gram-positivos que contém ácidos graxos cíclicos como principal componente da membrana celular e são formadores de esporos, sendo estes os principais fatores que propiciam sua resistência aos processos térmicos empregados em alimentos. O pH de crescimento varia de 2,2 a 6,0 e a temperatura de 35 ºC a 55 ºC. Essas bactérias podem alterar as características sensoriais de alimentos como sucos cítricos, pois produz um composto chamado guaiacol (2-methoxyphenol) caracterizando odor e sabor desagradáveis ao produto. Estes micro-organismos frequentemente são associados à deterioração de sucos de laranja reconstituídos e como este gênero está amplamente distribuído nos solos, é inevitável que a fruta carregue essa bactéria ou o seu esporo para o ambiente industrial. O suco de laranja é o único produto brasileiro que detém mais de 50% da produção mundial, além de 85% das exportações, portanto contribui significativamente para a balança comercial do país e sua deterioração acarreta sérios prejuízos ao comércio. Atualmente, é difícil desenvolver uma metodologia padrão para a determinação e identificação de *Alicyclobacillus* spp., por causa das diferentes faixas de pH e temperaturas ótimas para crescimento das diferentes espécies. Para o controle de bactérias no processamento do suco a indústria utiliza procedimentos como a lavagem das frutas, aspersão de sanitizantes e a pasteurização do produto. Porém, tanto o esporo como o biofilme podem resistir a todos esses procedimentos, podendo a bactéria estar presente no produto final. Alguns fatores como a concentração e o tempo de contato entre o sanitizante e o micro-organismo interferem na redução das bactérias, no entanto, não existem dados conclusivos sobre a ação dos sanitizantes utilizados para a lavagem das frutas em esporos e biofilme de *Alicyclobacillus* spp.

OBJETIVOS. Os objetivos deste trabalho foram: Avaliar a recuperação e o crescimento de três espécies de *Alicyclobacillus (A. acidoterrestris, A. acidocaldarius e A. pomorum-like*) nos meios mais utilizados encontrados na literatura: ALI ágar (*Alicyclobacillus* medium), BAT (*Bacillus acidoterrestris* thermophilic agar), K-ágar e YSG ágar (Yeast extract starch glucose); Avaliar a aderência e formação de biofilme de *Alicyclobacillus acidoterrestris* em superfícies do processamento industrial do suco de laranja (aço inoxidável, PVC - policloreto de polivinila - e nylon) e Avaliar a eficácia dos sanitizantes ácido peracético, hipoclorito de sódio e quaternário de amônia na remoção do biofilme formado e na inativação dos esporos deste micro-organismo.

MATERIAIS E MÉTODOS. Duas espécies (*A. acidoterrestris* DSM 3922^T e *A. acidocaldarius* DSM 446^T) foram obtidas junto à Coleção de Culturas Alemã de Micro-organismos e Culturas Celulares (DSZM – Deutsche Sammlung Von Mikroorganismen und Zellkulturen), e também foram utilizadas três espécies de *Alicyclobacillus* originárias de indústrias de suco concentrado de laranja, identificadas e estocadas na Coleção Brasileira de Micro-organismos de Ambiente e

Indústria – CBMAI, Campinas SP: *A. acidoterrestris* – CBMAI 0281; *A. acidocaldarius* – CBMAI 0294 e *A. pomorum-like* – CBMAI 0278.

Os meios de cultura testados foram preparados de acordo com o procedimento indicado pela legislação e encontrados na literatura, sendo que o pH de todos os meios foi padronizado para 4,0. Os meios testados foram: ágar (*Alicyclobacillus* medium), BAT (*Bacillus acidoterrestris* thermophilic agar), K-ágar e YSG ágar (Yeast extract starch glucose). As superfícies testadas para a produção de biofilme foram: cupons de aço inox AISI 304 (1,0 x 1,0 x 0,1 cm), PVC – policloreto de polivinila (1,0 × 1,0 × 0,1 cm) e cerdas de Nylon (1,0 × 0,1 X 0,1 cm), sendo todas as superfícies lavadas, individualmente, higienizadas e esterilizadas. Foram selecionados três agentes químicos: Ácido Peracético, Hipoclorito de Sódio e Quaternário de Amônia. Primeiramente, foi realizado a enumeração das linhagens-referência e os isolados nos quatro meios de cultura selecionados, através de plaqueamento em superfície e encubação das placas a 45 ºC por 5 dias. Foi realizada também a enumeração de *Alicyclobacillus acidoterrestris* com enriquecimento prévio em suco de laranja reconstituído. O *A. acidoterrestris* foi inoculado em tubos contendo as diferentes superfícies, para verificar a aderência de células à superfície e posteriormente foi realizado o tratamento com sanitizantes. Em seguida foi realizada a quantificação através do desprendimento das células após sonicação e plaqueamento para contagem. A aderência e a ação dos sanitizantes também foram verificadas através de microscopia de varredura. A determinação da concentração esporocida mínima para cada sanitizante também foi determinada através da técnica de microdiluição. Para verificar a ação dos sanitizantes na membrana celular da bactéria, foi realizada a Citometria de fluxo com iodeto de propídio. Todos os ensaios foram realizados em triplicata. Os dados foram analisados estatísticamente utilizando o software Assistat 7.6 Beta. Um valor de probabilidade de $p < 0,05$ foi aceito como indicando diferença significativa.

RESULTADOS E DISCUSSÃO. Nos ensaios de crecimento nos meios de cultura, o plaqueamento no meio K ágar apresentou uma menor recuperação dos micro-organismos quando comparado com os outros três meios de cultura. Para a recuperação das linhagens de *A. acidocaldarius* e *A. pomorum-like* houve diferença significativa para o meio K ágar quando comparados aos demais meios. Para os ensaios com *A. acidoterrestris*, realizados nos tempos 0h e após 24 horas de enriquecimento em suco a 45 °C não houve diferença significativa entre a contagem de colônias nos diferentes meios de cultura, também não houve diferença significativa entre os métodos de plaqueamento (superfície e profundidade) utilizados nos ensaios. As características encontradas nas colônias dos meios ALI, BAT e YSG foram semelhantes, apresentando colônias circulares, opacas, com bordas regulares de cor creme clara, sendo que o YSG apresentou também algumas colônias um pouco translúcidas. As colônias no meio K ágar apresentaram características morfológicas diferentes dos outros meios de cultura, apresentando colônias com bordas irregulares e algumas colônias translúcidas.Para os ensaios de aderência e formação de biofilme, o biofilme formado nas superfícies de aço e nylon apresentaram uma aderência maior de células do que o PVC em todas as condições experimentais. Através da microscopia eletrônica de varredura, observou-se a formação de um biofime nas superfícies de aço e nylon. Na superfície de PVC verifica-se a aderência de células e produção de extrapolissacarídeos, embora a contagem não tenha atingido 5.0 Log UFC/cm^2 após cinco dias de incubação. Com relação ao tratamento com sanitizantes nas superfícies após a aderência das

células, todos os tratamentos apresentaram diferenças significativas do controle, exceto o tratamento com 1000 ppm de hipoclorito de sódio nas superfícies de PVC e nylon que não apresentaram uma redução significativa das células aderidas. O tratamento com 1000 ppm de ácido peracético demonstrou uma eficácia maior, pois reduziu mais de 2 Log UFC/cm^2 em todas as superfícies. Já com relação aos ensaios realizados em esporos, o sanitizante que demonstrou melhor atividade esporicida foi o quaternário de amônia, que após 10 minutos de contato a concentração de 40,35 ppm já inativou completamente os esporos. O quaternário de amônia inativou tanto o esporo quanto a célula vegetativa com menores concentrações, no entanto essas baixas concentrações não foram eficientes na inativação do biofilme de *A. acidoterrestris.* Os gráficos de citometria de fluxo sugerem que o principal mecanismo de ação desses sanitizantes tanto na célula vegetativa quanto no esporo de *A. acidoterrestris* não acontece devido ao rompimento da membrana, pois o iodeto de propídio teve baixa fixação nas estruturas do micro-organismo após o tratamento com os sanitizantes.

CONCLUSÕES. A comparação entre os meios ALI, BAT, K ágar e YSG demonstrou que os meios ALI, BAT e YSG conseguiram recuperar a população inicial dos diferentes inóculos, não existindo diferenças significativas entre os resultados, apontando como vantagem a facilidade do preparo dos meios ALI e YSG, quando comparado ao meio BAT. O meio K ágar apresentou recuperação inferior para todos os inóculos, existindo diferença significativa para a recuperação de *Alicyclobacillus acidocaldarius* CMAI 0298^T e *Alicyclobacillus pomorum-like* CBMAI 0278, demonstrando que este meio em pH 4,0 não é o ideal para a recuperação de aliciclobacilos em suco de laranja com plaqueamento em superfície e incubação a 45 °C por 72 a 120 horas.Com base nos resultados de quantificação de células e microscopia eletrônica de varredura, verifica-se que houve a aderência e formação de biofilme por *A. acidoterrestris* nas superfícies de aço inoxidável, PVC e nylon. Os sanitizantes testados não foram eficientes na remoção total de células do biofilme de *A. acidoterrestris* nas condições avaliadas. O sanitizante que apresentou a maior redução no número de células foi o ácido peracético. Para todos os sanitizantes testados, o dobro da concentração que é bactericida para a célula vegetativa conseguiu apenas reduzir aproximadamente 2 Log UFC/cm^2 após a aderência e formação de biofilme nas superfícies. A melhor atividade esporicida foi realizada pelo quaternário de amônia, que em baixas concentrações e tempo de contato conseguiu inativar os esporos. A sanitização com ácido peracético e quaternário de amônia é um grande aliado contra *Alicyclobacillus acidoterrestris* para a indústria, no entanto esses sanitizantes dificilmente são aplicados em um mesmo processo de higienização, devido aos custos e praticabilidade. Para melhores resultados, as concentrações recomendadas dos produtos sanitizantes utilizados pela indústria devem basear-se em condições mais severas, levando em consideração os mecanismos de resistência específicos de cada bactéria como formação de biofilme e esporos.

Palavras-chave: *Alicyclobacillus*, meio de cultura, esporos, sanitizante, biofilme, suco de laranja.

ARTICLE 1

Evaluation of *Alicyclobacillus* spp. growth enriched in orange juice in different culture media

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Abstract

Bacteria of the genus *Alicyiclobacillus* spp. form spores and develop in acid media with the subsequent spoilage of citric juices. Since Brazil is the largest orange juice exporter in the world, the genus *Alicyiclobacillus* spp. has been studied extensively due to the changes in taste and smell which it causes. Several investigations have been reported on different culture media to identify and detect *Alicyiclobacillus* spp. Current analysis evaluates the recovery of spores of *Alicyiclobacillus* spp. by ALI, BAT, K agar and YSG media through the methodology suggested by ABECitrus. Five strains were used, two from reference strains and three from pasteurized concentrated orange juice. Cell recovery after the enrichment of reconstituted orange juice was also analyzed. An initial population of 6 log CFU/ml was inoculated and ALI, BAT and YSG media recovered this population within the different strains. There were no significant differences among the results although, when compared to BAT medium, preparation of ALI and YSG media was easier and thus more advantageous. K agar medium caused a lower recovery for all inocula with a significant difference only for *Alicyclobacillus acidocaldarius* 0298^T and *Alicyclobacillus pomorum-like* CBMAI 0278, in which recovery achieved was 3.66 and 4.11 log CFU/ml respectively.

Keywords: *Alicyclobacillus*, culture media, spores, orange juice.

Resumo

As bactérias do gênero *Alicyiclobacillus* spp. formam esporos e se desenvolvem em meios ácidos, podendo causar deterioração em sucos cítricos. O Brasil é o maior exportador de suco de laranja concentrado do mundo, portanto, este gênero vem sendo estudado por causar alterações de odor e sabor. Vários estudos relatam diferentes meios de culturas empregados para a detecção e enumeração de *Alicyclobacillus* spp., portanto este estudo teve como objetivo avaliar a recuperação de esporos de *Alicyclobacillus* spp. nos meios ALI, BAT, K ágar e YSG, utilizando a metodologia indicada pela ABECitrus. Cinco isolados diferentes foram utilizados, sendo dois de linhagens-referência e os outros três isolados de suco concentrado de laranja pasteurizado. Também foi verificada a recuperação das células após o enriquecimento em suco de laranja reconstituído. Foi inoculada uma população inicial de 6 log UFC/ml, sendo que os meios ALI, BAT e YSG conseguiram recuperar esta população nos diferentes isolados, não existindo diferenças significativas entre os resultados, apontando como vantagem a facilidade do preparo dos meios ALI e YSG, quando comparado ao meio BAT. O meio K ágar apresentou recuperação inferior para todos os inóculos, porém houve diferença significativa apenas para *Alicyclobacillus acidocaldarius 0298*^{T} e *Alicyclobacillus pomorum-like* CBMAI 0278, onde a recuperação foi de 3,66 e 4,11 log UFC/ml, respectivamente.

Palavras-chave: *Alicyclobacillus*, meios de cultura, esporos, suco de laranja.

Introduction

The genus *Alicyclobacillus* ssp. comprises bacteria which grow in acid media and at high temperatures. Since they may multiply in different types of food with pH lower than 3.7, they cause spoilage mainly in tomatoes, citric fruits and their derivates, such as fruit juices (HIPPECHEN et al. 1981). Brazil is the largest world exporter of concentrated orange juice with exports amounting to 400.000 tons in 2011 (CITRUS BR, 2012). This fact triggered several studies on new alternatives to avoid juice spoilage by bacteria such as those of the genus *Alicyclobacillus* spp.

Alicyclobacillus spp. are spore-forming bacteria and, therefore, resistant to pasteurization employed in juice processing and in the reconstitution of concentrated juice. After pasteurization, the spores may germinate and grow, change the orange juice's sensorial features, such as smell and flavor, and, in some cases, form slight sedimentation and turbidity in the product (SILVA and GIBBS, 2001). Species of *Alicyclobacillus*, such as *A. acidoterrestris* and *A.acidocaldarius*, may exhale an anti-septic odor due to the formation of guaiacol by the microorganism (GOCMEN et al. 2005).

Alicyclobacillus spp. are widely extant in soil and they may be detected by isolation and culture methodologies related to specific nutritional and mineral requirements (MOTOHIRO and HIROKO, 1994). Detection is conducted in several ways since the spores' thermal resistance may be affected by composition, ions, pH and organic acids (PONTIUS et al. 1998).

Due to different pH bands and temperatures for the growth of the different species, actually it is highly difficult to identify a standard methodology to assess the development of *Alicyclobacillus* spp. The culture media were formulated to detect specific species and are used to detect all kinds *Alicyclobacillus* spp. (MURRAY et al. 2007).

Current analysis evaluated the recovery and growth of three *Alicyclobacillus* species *(A. acidoterrestris*, *A. acidocaldarius* and *A. pomorum-like*) in the most used media reported in the literature, or rather, ALI agar (*Alicyclobacillus* medium), BAT (*Bacillus acidoterrestris* thermophilic agar), K-agar and YSG agar (Yeast extract starch glucose) under the same temperature and pH conditions.

Materials and methods

Microbial strains

Standard strains of the species *A. acidoterrestris* and *A. acidocaldarius* were provided by the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen or DSZM) and isolates employed were deposited at the Brazilian Collection of Environment and Industry Microorganisms (CBMAI) at the Multidisciplinary Center of Chemical, Biological and Agricultural Research (CPQBA / UNICAMP), according to identification below:

- *A. acidoterrestris* DSM 3922^T - (CBMAI: 0244^T) – Origin: Soil
- *A. acidocaldarius* DSM 446^T – (CBMAI: 0298^T) – Origin: Soil

Three species of *Alicyclobacillus* from orange juice concentrate industries were also retrieved, identified and stored at the Brazilian Collection of Environment and Industry Microorganisms (CBMAI)**,** Campinas SP Brazil:

- *A. acidoterrestris* – CBMAI 0281
- *A. acidocaldarius* – CBMAI 0294
- *A. pomorum-like* – CBMAI 0278

The strains were kept at -80 ºC at the Laboratory of Water, Environment and Food Microbiology of the State University of Maringá, PR Brazil.

Concentrated orange juice

Concentrated orange juice samples from an exporting company in the region of Paranavaí PR Brazil were used in current analysis. Samples were previously certified on the absence of *Alicyclobacillus*. Assay sample was produced on the 6th October 2010 with Brix 65.81° and stored at -18 °C till analysis. The juice was then reconstituted aseptically for 12°Brix with sterile distilled water.

Composition of culture media

Tested culture media were prepared according to methods found in several studies and the pH of the media was standardized at 4.0, according to components described in Table 1.

Preparation of BAT medium (DEINHARD et al., 1987)

BAT medium was prepared by weighing all components in 500 ml distilled water, with the exception of the agar. Afterwards, pH was adjusted to 4.0 by 1N solution of H_2SO_4 or 1N solution of NaOH. Separately prepared in 500 ml distilled water, the still hot agar was added to the already autoclaved medium components, in a sterile environment, and medium was immediately poured on the plates.

Formulation of trace B was given in g/L of distilled water with 0.66 $CaCl_2$ x $2H_2O$; 0.18 $ZnSO_4$ x $7H_2O$; 0.16 $CuSO_4$ x $5H_2O$; 0.02 $MnSO_4$ x $4H_2O$; 0.18 $CoCl_2$ x $6H_2O$; 0.10 H_3BO_3; and 0.3 Na_2MoO_4 x $2H_2O$. Solution was sterilized by filtration after the above preparation.

Preparation of K agar medium (WALLS and CHUYATE, 2000)

Components of K agar medium and agar were weighed in a single erlenmeyer flask. After autoclave of the medium, pH was adjusted aseptic to 4.0, by an aqueous solution of malic acid

25%, sterilized by filtration, and measured by pH tape. The still hot medium was immediately poured on the plates.

Preparation of ALI medium (WISSE and PARISH, 1998) **and YSG medium** (MOTOHIRO and HIROTO, 1994)

ALI and YSG and media was prepared by weighing all components in 500 ml distilled water, with the exception of the agar; pH was then adjusted to 4.0 with a 1N solution of H_2SO_4 or 1N solution of NaOH. Agar was prepared separately in 500 ml of distilled water.

Preparation of spores' standard solutions

Standard suspensions of reference strain spores and of isolates of orange juice were prepared and maintained under refrigeration at 4°C to be used as inoculants in the assays. Al least five isolated colonies were collected by a sowing loop from cell culture in BAT (agar) medium and then transferred to tubes with 3 ml BAT medium (broth). Suspension was incubated at 45^{o}C during 24 h after 0.3 ml were re-suspended in steril tube with 10 ml of BAT broth and once more incubated at 45^{o}C for 72 h. The culture was then transferred to a cryotube and centrifuged at 9500 x g for 10 min. Precipitate was cleansed thrice with sterilized distilled water and stored at 4°C.

Recovery of reference strains and isolates of orange juice in the four culture media

Spore suspensions standardized with 6 log CFU/ml of reference strains CBMAI 0244^{T} and CBMAI 0298^{T} and isolates CBMAI 078, CBMAI 0281 and CBMAI 0294 were re-suspended in sterile distilled water and underwent a thermal shock of 80°C during 10 min. Moreover, 100µL were inoculated in duplicate by surface plating on the above-mentioned four culture media and spread with a Drigalski loop. Plates were incubated at 45°C during 5 days and

results monitored during 3 and 5 days of growth. Checking of colonies was performed by gram- and spore-staining (Wirtz-Conklin staining technique) to verify the morpho-staining characteristics by microscopy.

Recovery of *Alicyclobacillus acidoterrestris* with enrichment in reconstituted orange juice in four culture media

Suspension of spores CBMAI 0244T was diluted in saline and once in orange juice with 12°Brix. . The juice containing spores underwent a thermal shock at 80 °C for 10 min. It was then plated in the four culture media by surface and deep plating methods, in duplicate, for each method. The plates were then incubated at 45 °C during 72 h. Juice with spores was incubated during 24 h for germination. The analysis procedure was repeated after 24 h for growth after enrichment in the orange juice. Checking of colonies was performed by gram- and spore-staining (Wirtz-Conklin staining technique) to verify the morpho-staining characteristics by microscopy.

Statistical analysis

Three independent assays were undertaken in duplicate. Mean of results related to microbiological counts was tested by analysis of variance ANOVA and significant difference was confirmed by Tukey's test at $\alpha=0.05$.

Results

An initial population of 6 log CFU/ml of each micro-organism was inoculated in different media to verify its recuperation. Graph 1 shows the results of the recuperation of reference strains and pasteurized orange juice isolates in different culture media.

No significant difference existed among culture media for assays with CBMAI 0244^{T}, CBMAI 0281 and CBMAI 0294. Since germination in K agar medium had lower results when compared to that of the other three culture media, low recuperation of microorganisms was detected in this medium.

Significant differences existed for K agar medium in the case of the recuperation of strains CBMAI 0298^{T} and CBMAI 0278 when compared to that of other culture media. K agar had a low recuperation of the microorganism's cells.

An initial spore population of 2 log CFU/ml was used for inoculation in reconstituted orange juice. A recovery of up to 2 log CFU/ml was achieved after thermal shock, plating and incubation at 45ºC. There was a recovery of 3 log CFU/ml after juice incubation with inoculum for 24 h at 45ºC. Graphs 2 and 3 show results for inoculation respectively by surface and deep methods in *Alicyclobacillus acidoterrestris* spores, enriched in orange juice reconstituted in times 0h and 24 h after shock, at 80°C, during 10 min.

There was no significant difference between colony counts in different culture media for assays at time 0h. Similarly there was no significant difference between (surface and deep) plating methods used in the assays. However, K agar medium had a lower performance in the recovery of the colonies. No significant difference existed among the four media and between the plating methods in assays undertaken after 24 h enrichment in juice at 45^{o}C.

Monitoring of plates after the third day of incubation showed a substantial growth of the colonies in media ALI, BAT and YSG for all assays. Whereas no colony growth occurred in K agar medium till the third day of incubation, lower counts than those from other culture media were reported after the fifth day of incubation. K agar's different composition may have not favored the fast development of *Alicyclobacillus* cells.

The Fig. 1 showed the colonies' morphological characteristics after the 72 h-incubation at 45°C. Colonies' characteristics in media ALI, BAT and YSG were similar, or rather, circular

and opaque colonies with regular light cream edges. YSG also revealed somewhat translucid colonies. K agar medium had different morphological characteristics from the other culture media and revealed irregular edges and some translucent colonies.

Gram-positive and spore-staining confirmed the morpho-staining characteristics of *Alicyclobacillus* colonies which grew in the four culture media. Gram-positive bacilli with sub-terminal spores, characterizing inoculated colonies, were reported.

Discussion

Results for the four media evaluated showed a good recovery of micro-organisms in direct inoculation and enrichment with reconstituted juice. However, K agar results were lower than those of the others in the two assays.

According to the International Federation of Fruit Juice Producers (IFU, 2007), when K agar medium was incubated at 45°C, the growth of several alicyclobacilli species was constrained and only the growth of *A. acidoterrestris* was provided. However, the American Public Health Association (APHA) suggested K agar medium at pH 3.7 and 3-day incubation at 43°C. However, in our experiments, the K-agar medium was incubated at 45 ° C and colonies showed only after the fifth day for all species.

PARISH and GOODRICH (2005) reported that ALI medium significantly recovered a larger number of *Alicyclobacillus* than K agar medium, which was corroborated in current study.

According to methodology by WISSE and PARISH (1998), pH should be adjusted at 3.5 for the preparation of ALI medium, although the standard pH at 4.0 did not affect results which were satisfactory when compared to those in BAT medium whose preparation with pH adjustment at 4.0 was described by DEINHARD et al. (1987).

When WITTHUHN (2007) evaluated the media Potato Dextrose Agar (PDA), Orange Serum Agar (OSA), *Bacillus acidocaldarius* medium (BAM), YSG and K agar for alicyclobacilli

growth, the author reported that media PDA and OSA provided the best cell recovery. K agar medium failed to recover *A. acidoterrestris* cells inoculated in concentrated pear juice and failed to recover *A. pomorum* cells inoculated after thermal treatment at 80°C for 10 min.

According to MURRAY et al. (2007), the comparison of several culture media revealed that BAT medium tended to provide the best results and justified the IFU's suggestion (2007) to use the medium to detect *Alicyclobacillus* in fruit juices. Results by MURRAY et al. (2007) were corroborated by current analysis, even though ALI and YSG media results were similar to those by BAT.

With regard to the isolation and counting method by plating for spore recovery, no significant difference existed between surface and deep methods, although studies by PETTIPHER et al. (1997) and MURRAY et al. (2007) showed that surface method provided higher results when compared to those by deep method. Colonies sown deep in the medium were frequently overlain by others and were smaller than colonies inoculated by surface method, which made difficult colony counting. The observation of the colony's characteristics and the recovery of isolates for identification were easier by surface method, also due to the microorganisms' physiological characteristics.

Results in current analysis indicated a significant difference for the growth of *A. acidocaldarius* in K agar medium and corroborated results by MURRAY et al. (2007), who compared the recovery among K agar, BAT and ALI media. The recovery of the bacterium in K agar medium was significantly less when compared to that in other media. FARRAND et al. (1983) showed that higher incubation temperatures (55°C) favored *A. Acidocaldarius* growth. However, temperature was 45ºC and *A. acidocaldarius* recovery was higher when compared to that of *A. acidoterrestris* not only in the inoculation of reference strains but also in the recovery of pasteurized orange juice isolates. This fact suggested that temperatures much higher than 43°C were not required for the development of *A. acidocaldarius*.

ABREU FILHO (2005) characterized the taxonomy of thirty strains of Alicyclobacilli isolates in the orange juice process, with special attention to CBMAI 0278, CBMAI 0281 and CBMAI 0294 used in current study. In their study, the species A. pomorum-like showed no sensory changes in orange juice, although this species have shown a good recovery in our experiments, it does not present a great potential for deterioration as the other species used in this study.

Conclusions

Comparison among ALI, BAT, K agar and YSG media showed ALI, BAT and YSG media recovered the initial population of the different inocula with no significant differences in results. The easy preparation of ALI and YSG media is an asset when compared to the preparation of BAT. K agar medium provided a lower recovery for all inocula with significant difference for the recovery of *A. acidocaldarius* CMAI 0298^{T} and *A. pomorum-like* CBMAI 0278 and showed this medium at pH 4.0 was not ideal for the recovery of alicyclobacilli in orange juice with incubation at 45°C, between 72 and 120 h.

Acknowledgements

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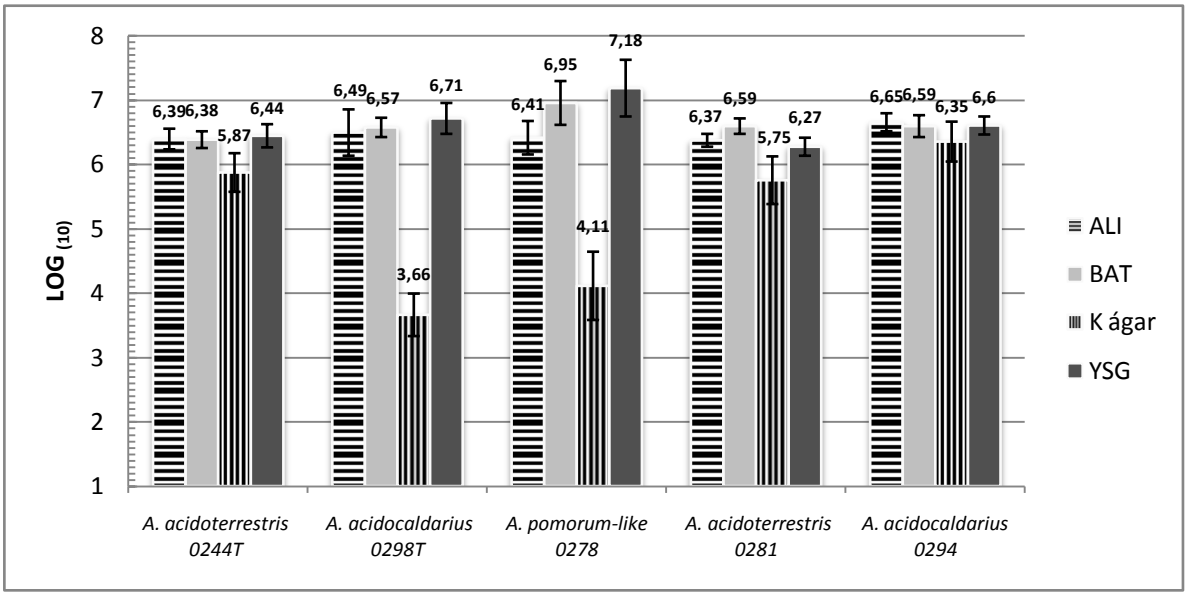
Table 1 – Formulation of culture media

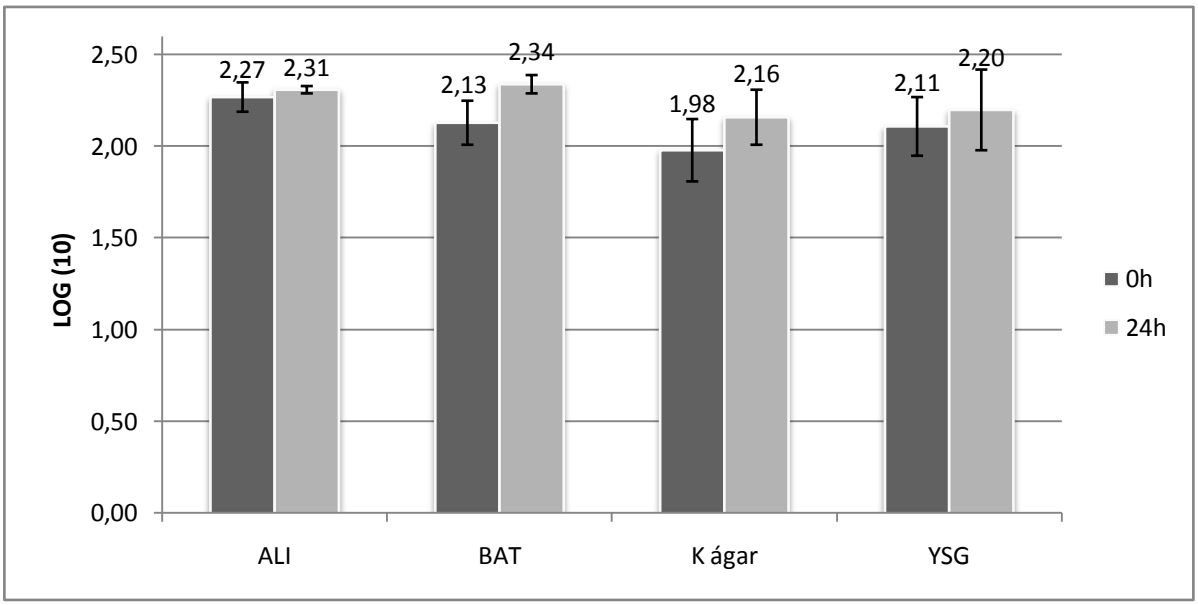
Components	BAT (g/l)	K agar (g/l)	YSG (g/l)	ALI (g/l)
Soluble starch (Dinâmica)	-	-	2	4
Yeast Extract (Merck)	2	2.5	2	4
Glucose (Nuclear)	5	1	1	2
$(NH_4)_2\ SO_4$ (Nuclear)	0.2	-	-	0.4
$Mg\ SO_4\ .\ 7H_2O$ (Synth)	0.5	-	-	1
$CaCl_2\ .\ 2H_2O$ (Nuclear)	0.25	-	-	0.5
KH_2PO_4 (Merck)	3	-	-	6
Peptone (Merck)	-	5	-	-
Tween 80 (Sigma)	-	1	-	-
Trace B*	1	-	-	-
Agar (Difco)	20	20	20	20

Trace B*: Solution of salts in trace elements (FARRAND et al., 1983)

Graph 1 – Results for reference strains and isolates of pasteurized orange juice.



Graph 2 – Results for *Alicyclobacillus acidoterrestris* growth enriched in reconstituted orange juice at 0h and 24 h plated by surface method.



Graph 3 – Results for *Alicyclobacillus acidoterrestris* growth enriched in reconstituted orange juice at 0h and 24 h plated by deep method.

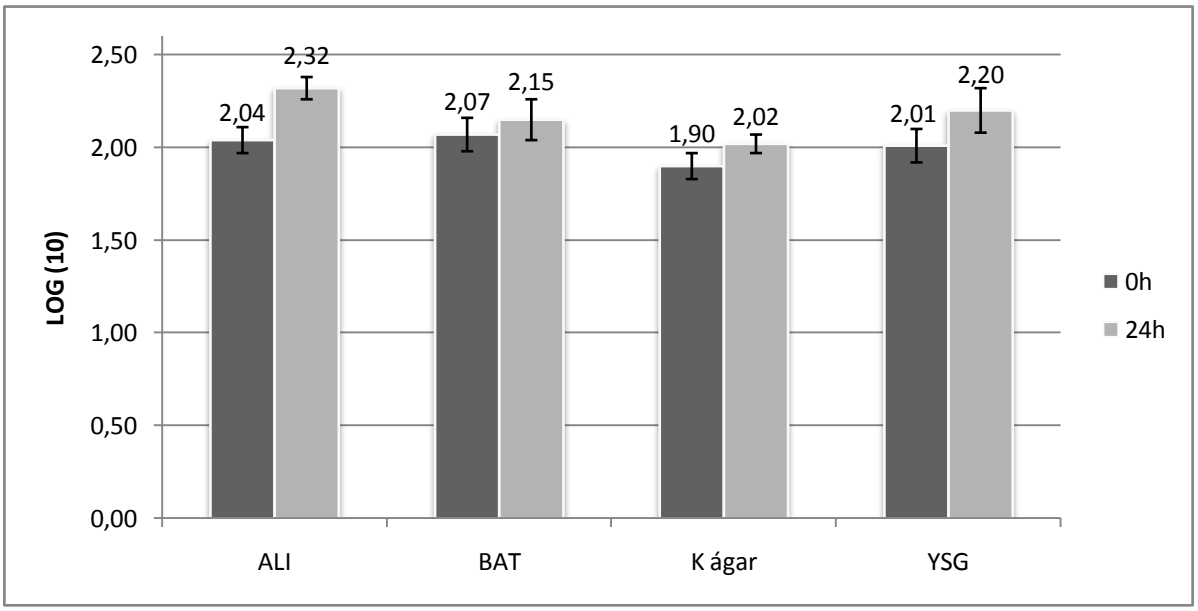
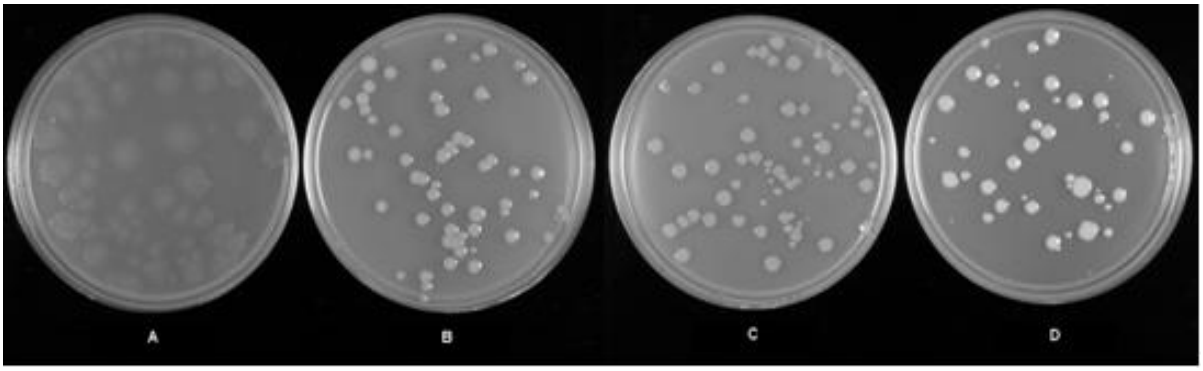


Figure 1 – *Alicyclobacillus acidoterrestris* colonies in: (A) K agar, (B) ALI media, (C) YSG and (D) BAT ágar after 72 h at 45°C.



ARTICLE 2

Sanitizers on *Alicyclobacillus acidoterrestris*

The resistance of *Alicyclobacillus acidoterrestris* spores and biofilm to industrial sanitizers

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KEYWORDS: *Alicyclobacillus,* sanitizer, biofilm, orange juice, spore.

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ABSTRACT

This study evaluated the adhesion and biofilm formation of *Alicyclobacillus acidoterrestris* on industrial orange juice processing surfaces and the bactericidal efficacy of peracetic acid, sodium hypochlorite and quaternary ammonia after biofilm formation. The efficacy of these sanitizers on the spores of this microorganism was also evaluated. Stainless steel and nylon surfaces exhibited higher cell adhesion levels than did polyvinyl chloride surfaces. Peracetic acid was the most effective in removing biofilms from all surfaces ($p<0.05$) and also reducing bacterial counts to 3 logs on the surface of PVC, but the other sanitizers also reduced the bacterial counts by 2 log CFU/ml. Quaternary ammonia exhibited the optimal minimum sporicidal concentration, preventing spore germination after only 15 seconds of contact at a concentration of 82 ppm. Flow cytometry indicated that the spores and cells had low incidences of plasma membrane lysis after treatment with sanitizer, suggesting that lysis is not the principal mode of action for these sanitizers on *A. acidoterrestris*.

The genus *Alicyclobacillus* spp.is composed of gram-positive bacilli containing cyclic fatty acids as a major component of the cell membrane that forms spores, making them resistant to the thermal processes used during food processing. The pH required for growth ranges from 2.2 to 6.0,whilethe temperature can range from 35°C to 55°C. This bacteria produces some components, such as 2,6-dibromophenol, 2,6-dichlorophenol and 2-methoxyphenol, the latter known as guaiacol, that gives food products an unpleasant smell and taste with medicinal or antiseptic characteristics of such foods as citrus juices (*15, 20, 22, 23*).

Through the presence of spores in juice concentrate, these microorganisms are frequently linked to the spoilage of reconstituted orange juice that has been stored at high temperatures after pasteurization (*3, 20*). This genus is widely distributed in the soil; thus, fruit will inevitably carry the bacteria or spores to the industrial processing plant. The first case of juice spoilage caused by a spore-forming acidophile was reported for apple juice (pH 3.15) bottled aseptically in Germany in 1982 (*3*).

Orange juice is the only product for which Brazilian production accounts for more than 50% of worldwide supply and accounts for 85% of the country's exports. Orange juice even surpasses the production of coffee, beef, chicken and sugar (*4*). In addition to being a source of pride for the country, citrus production brings in billions of dollars to Brazil's economy via exports. Thus, orange juice concentrate exports significantly contribute to the country's trade balance, and product spoilage can cause severe economic damage.

To control bacteria during juice processing, manufacturers swash the fruit, spray them with sanitizers and extract and pasteurize the juice. However, both spores and biofilms can be resistant to these methods and remain in the final product. Spores in orange juice concentrate encounter conditions suitable for vegetative growth when the product is reconstituted and pasteurized (*3*).

Several factors, such as concentration and time of contact between the microorganisms and the sanitizer can affect the sanitizer's bactericidal ability; however,

conclusive data on the activity of the sanitizers used to wash the fruit against *Alicyclobacillus* spp. spores and biofilm are lacking.

Microbial biofilm formation is a multi-stage process in which cells adhere to a surface (initial reversible adhesion)and then produce an extracellular matrix (containing polysaccharides, proteins and DNA), leading to an irreversible adhesion(*19*). The cells in the biofilm exhibit coordinated group behavior that increases their resistance to sanitizers, making them a problem for the food industry (*9, 16*). However, studies on *Alicyclobacillus* spp. adhesion and biofilm production remain limited.

This study sought to evaluate *Alicyclobacillus acidoterrestris* adhesion and biofilm formation on orange juice processing surfaces (stainless steel, polyvinyl chloride [PVC] and nylon) and to measure the efficacy of peracetic acid, calcium hypochlorite and quaternary ammonia in biofilm removal and inactivation of the microorganism's spores.

MATERIALS AND METHODS

Microbial strain. The species *A. acidoterrestris* DSM 3922^T CBMAI 0244^{T}provided by the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen or DSZM) was used in this study. The bacteria were obtained by transferring cells from a BAT media plate (*8*) to a test tube containing 5 ml liquid BAT media. The suspension was incubated at 45°C for 72 h, or until a sporulation level of approximately 80% was observed in the cells using a phase contrast microscope. The culture was then transferred to a tube and centrifuged at 10,000 rpm for 1 min, and the pellet was washed 3 times with sterile distilled water. The pellet was later resuspended in 1 ml sterile distilled water, aliquoted into 2-mlNunccryotubes (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −20°C. Spore suspension viability was determined by dilution and plate counting in duplicate and was expressed in CFU/ml. The spore suspension aliquots were activated by thermal shock (80°C for 10 min in a water bath), diluted, plated on BAT

plates (Inlab Dispo-petri 15, Diadema, SP, BR) by streaking and incubated at 45°C for 24–48 h.

Experimental conditions. Square pieces of AISI 304 stainless steel, PVC (1.0 × 1.0 × 0.1 cm) and strands of nylon (1.0 × 0.1 × 0.1 cm) were used as test surfaces. The squares were individually washed, sanitized and sterilized using the procedure published by Marques et al. (*12*).Three chemical agents were selected and tested on vegetative *A. acidoterrestris* cells at concentrations previously determined using the MIC technique (*5*) and a contact time of 10 min. A 500 ppm peracetic acid solution was prepared from 17% peracetic acid (Ecoper, SP,BR), a 1000 ppm sodium hypochlorite solution was prepared from 15% sodium hypochlorite (Quibras, PR, BR),and a 15.62 ppm solution of quaternary ammonia-Benzalkonium chloride was prepared (Acros organics, New Jersey, USA).

Cell adhesion and quantification. The test squares were placed in sterile 24-well plates (TPP®, Switzerland, EU) containing 900 µl BAT broth at pH 4.0 (*8*) and 100 µl vegetative *A. acidoterrestris* cell suspension. The plates were incubated for 5 days at 45°C during which the BAT media was changed every 24 h. The squares were then treated with the sanitizers for 10 min then neutralized with one of the following: 0.2% sodium thiosulfate (Synth®, Diadema, SP, BR) was used to neutralize peracetic acid and sodium hypochlorite, while Letheen Broth (Difco® Laboratories, Detroit, MI) was used for the quaternary ammonia. The squares were subsequently transferred to cryotubes containing 1.0 ml saline solution and were sonicated for 5 min at 25000 hertz to disperse the cells. Serial dilutions were then made and plated onto BAT agar via streaking. The plates were incubated at 45°C for 48 h. After incubation, the cells were counted and the results expressed as log CFU/cm^2.

Scanning microscopy. Prior to analysis by scanning electron microscopy, the squares were treated with sanitizer for 10 min then neutralized. The squares were washed in saline solution and fixed in 2.5% glutaraldehyde (Sigma-Aldrich, St. Louis, USA) in a 0.1 M

sodium cacodylate buffer (EMS, Hatfield, PA).They were then dehydrated in ethanol, dried to the critical point in CO_2, coated with gold and examined under a Shimadzu SS-550 scanning electron microscope (*11*).

Determination of the minimum sporicidal concentration for each sanitizer. The sporicidal concentration was determined by microdilution in BAT media in a 96-well plate (TPP®,Switzerland,EU). The sanitizers were serially diluted in the wells, and 5 µl spore suspension standardized to 4 log was added to each well. The contact times of 15 s, 1, 5, 10, 15 and 30 min of the sanitizer with the spore suspension was also evaluated. After each contact time, 100 µl of the neutralizing solution was added. The plate was then sealed and placed in an 80°C water bath for 10 min. After removal from the water bath, 10 µl from each well was plated on BAT agar in triplicate. The plates were incubated at 45°C for 24 h. The minimum sporicidal concentration is defined as the lowest concentration that results in a negative subculture on the BAT agar.

Flow cytometry. *A. acidoterrestris* spores and vegetative cells at a standardized 4 log concentration were treated with sanitizers at their respective MIC concentrations for 10 min, and then neutralized. For cases in which the MIC concentration was not found for the spores during the contact time, as with peracetic acid or sodium hypochlorite, the maximum evaluated concentration (1000 ppm) was used. The spore suspensions were then subjected to thermal shock at 80°C for 10 min. The cells were resuspended in phosphate buffered saline (PBS) buffer, centrifuged, washed and stained with propidium iodide in PBS (2µg/ml). The samples were counted in a FACSCalibur (BD Bioscience) flow cytometer, and the bacterial survival rates following sanitizer treatment were determined using fluorescence intensity.

Statistical analysis. All assays were performed in triplicate. The cell counts in the biofilms were compared using Student's t-test. The data were analyzed using the Assistat 7.6

Beta software package. A probability of $p<0.05$ was used to determine the significant differences.

RESULTS AND DISCUSSION

The quantification of *A. acidoterrestris* cells adhered to the various surfaces and the counts after the 10min sanitizer treatments are shown in Table 1.

PVC is the material used on the mats of the food industries. In the processing of orange, it is used to transport the oranges to the extractor. Nylon is applied to the bristles that are used in washing the fruit, and stainless steel is present throughout the processing, in silos, the extractor and also the storage of the juice. Biofilm formation on these surfaces helps to the bacteria that are present throughout the industrial environment.

Steel and nylon exhibited greater cell adhesion than PVC under all experimental conditions. Ronner and Wong (*18)* and Wirtanen, Ahola and Mattila-Sandholm (*21*) demonstrated that a minimum of 5.0–6.0 log CFU/cm^{2}is necessary for biofilm formation, and lower counts may indicate adhesion only. Scanning electron microscopy (Figure 1) confirmed the biofilm formation on the steel and nylon surfaces. We also observed cell adhesion and extracellular polysaccharide production on the PVC surface but the cell counts did not reach 5.0 Log CFU/cm^{2}even after five days of incubation.

The literature lacks information on biofilms produced by *A. acidoterrestris* on stainless steel, PVC and nylon, making comparisons of our results with previously published studies difficult.

All of the sanitizer treatments were significantly different from the control, except the treatments of PVC and nylon with the MIC concentration of sodium hypochlorite, which did not significantly reduce the number of adhered cells.

Treatment with 2× MIC peracetic acid reduced the cell counts by more than 2 Log CFU/cm^{2}on all surfaces. Similar results were obtained by Meira et al. (*13*), who evaluated the

use of peracetic acid and sodium hypochlorite on biofilms produced by *Staphylococcus aureus* on stainless steel and polypropylene.

Moretro et al. (*14*) tested nine commercial disinfectants on *Salmonella* spp. biofilms on stainless steel at the concentrations recommended by the manufacturers. These researchers also demonstrated that sodium hypochlorite-based solutions were the least effective followed by quaternary ammonia-based compounds, while peracetic acid solutions reduced counts by more than 4 logs.

Cruz and Fletcher (*7*) tested seven groups of commercial sanitizers with different active ingredients on *Listeria monocytogenes* biofilms grown on a polyvinyl surface for 48 h. The MIC values for the biofilms were much higher than those for the bacterial suspensions, regardless of the active compound in the solution. Our results were similar; peracetic acid was the only sanitizer with a MIC value lower than that recommended by the manufacturer (500 ppm). The quaternary ammonia based-compounds had a MIC value two times higher than that recommended (2000 ppm), and the sodium hypochlorite-based solutions had a MIC value eighteen times higher than the recommended concentration (3600 ppm).

Figure 1 shows that peracetic acid removed the polysaccharide matrix more efficiently from the three surfaces, leaving only a small number of adherent cells. Treatment with sodium hypochlorite only slightly affected the biofilm matrix, resulting in cell resistance, similar to, but less intense than, that with quaternary ammonia.

According to Abee et al. (*1*), biofilms provide an ideal environment for bacillus sporulation, and this process is tightly linked to the development of the biofilms, as spores appear in their upper structures.

According to a study conducted by Podolak et al. (*17*), products based on peracetic acid and sodium hypochlorite were more effective against spores of *Alicyclobacillus acidoterrestris* that the product chlorine dioxide and tested at temperatures of 40 ° C to 90 ° C in apple juice.

The apparent efficacy of the peracetic acid-based products on the biofilms is likely based on several factors including the ability of the small molecule to penetrate the

extracellular matrix of the biofilm, the mode of activity of the antimicrobial agent and its tolerance of moderate quantities of organic material (*2*).

In another study by Friedrich et al. (*10*), sodium hypochlorite also not had a good result on *Alicyclobacillus* spp. spores under the conditions tested. The authors tested the effectiveness of chlorine dioxide and sodium hypochlorite on spores on three surfaces: stainless steel, wood and rubber and the most effective concentration/time regime applied was 100 ppm of chlorine dioxide for 10 min.

The performance of a sanitizer can be affected by the biofilm stage and by the structure of the extracellular polysaccharide matrix. Cruz and Fletcher (*7*) demonstrated that the ability of *L. monocytogenes* strains to survive exposure to sanitizers may be more closely linked to the quantity of extracellular polysaccharide produced by the cells in the biofilm than to the number of cells in the biofilm, or even other factors such as the genetic subtype.

In this study, we found that the reduced number of cells attached in the *A. acidoterrestris* biofilms on PVC did not interfere with our analysis of the sanitizers, as the biofilms behaved similarly to those grown on steel and nylon with higher cell counts. This finding indicates that the quantity of cells in the biofilm does not directly affect the sanitizer function.

Scanning electron microscopy (Figure 1) indicated that surface type contributed to the cell adhesion. Steel provided a rougher surface, which facilitated adhesion. This result was also observed on the nylon strands, which provided pockets for biofilm development due to the space between the strands. The microscopy results are consistent with the cell survival assays, which also demonstrated reduced adhesion and biofilm formation on the PVC surface.

The presence of *A. acidoterrestris* spores in orange juice causes serious problems after its reconstitution and pasteurization, as the spore encounters conditions that are ideal for germination and multiplies. Spores that contaminate the final product may originate from biofilms that formed on the surfaces during industrial processing.

The sporicidal activities of the sanitizers, shown in the graphs of Figure 2, indicate that peracetic acid was less effective against the spores than the biofilm. Peracetic acid at a concentration of 1000 ppm (2 × MIC for the vegetative cells) and a 10 min spore contact time reduced the cell counts by 1 Log CFU/ml and the biofilm by more than 2 Log CFU/cm^2 on all surfaces. After 30 min of contact, this concentration of peracetic acid reduced the spore concentration by only 1.5 Log CFU/ml and did not completely inactivate the spore germination at any of the concentrations tested in the experiment.

The sanitizer that demonstrated the best sporicidal activity was the quaternary ammonia, which completely inactivated the spores after 5 min of contact at a concentration of 40.35 ppm. The quaternary ammonia inactivated both the spores and the vegetative cells at low concentrations (15.62 ppm), but these low concentrations did not effectively inactivate the *A. acidoterrestris* biofilms.

Sodium hypochlorite exhibited low activity on the biofilm and spores, although it was able to inactivate the spores at a concentration of 700 ppm after 30 min of contact. However, this combination of time and concentration is impractical.

The flow cytometry charts in Figure 3 suggest that the principal mechanism for these sanitizers on both the *A. acidoterrestris* vegetative cells and the spores does not rely on membrane lysis because the propidium iodide did not strongly stain the microorganism after sanitizer treatment.

However, little information is available in the literature on the mode of action for the sanitizers against *Alicyclobacillus* spp., and the present study is a pioneering work on *A. acidoterrestris*. Most previous studies suggest that the mode of action is similar to that reported for *Bacillus* spp. and *Clostridium* spp.

Cortezzo et al. (*6*) reported that the targets of the sanitizers may be the fatty acids and proteins in the spore, but their results obtained using *B. subtilis* indicated that oxidation of the unsaturated fatty acids did not play a significant role in spore inactivation. Thus, the target may be the proteins, as their membranes are rich in fatty acids and are not significantly affected by the sanitizers in the present study.

Although the exact nature of the damage inflicted by the sanitizers on the *Alicyclobacillus* spp. spores is unknown, Cortezzo et al. (*6*) found that the damaged spore could not germinate. If it did germinate, the membrane would have been so damaged that the cell would rapidly die.

The cell count and scanning electron microscopy results indicate that *A. acidoterrestris* adheres to and forms biofilms on stainless steel, PVC and nylon. The tested sanitizers did not effectively remove *A. acidoterrestris* in biofilms under the given conditions. The sanitizer that best reduced the cell numbers was peracetic acid. For all sanitizers, doubling the bactericidal concentration for vegetative cells only reduced the cell concentrations by 2 Log CFU/cm^{2}after adhesion and biofilm formation on all surfaces.

The quaternary ammonia exhibited the best sporicidal activity, as it inactivated the spores at low concentrations and short contact times. Sanitization using peracetic acid and quaternary ammonia is a good approach to control *Alicyclobacillus acidoterrestris* in industrial settings, but these sanitizers are difficult to apply together, due to their cost and practicality.

Considering the need to reduce or eliminate the amount of spores in the industrial environment, the daily use of sanitizing the basis of quaternary ammonia is the most indicated. To eliminate and prevent the formation of biofilms, the ideal is to establish a frequency of application of peracetic acid for all surfaces. Working in this way with these two sanitizers the industry can get good results.

For better results, the recommended concentrations of the sanitizers used in industry should be based on more stringent conditions, considering the specific resistance mechanisms for each bacterium, such as biofilm and spore formation.

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Figure Legends

FIGURE 1 – Scanning electron microscope images of steel, PVC and nylon surfaces after incubation with *A. acidoterrestris* at 45°C for 5 days followed by sanitizer treatment. Figures A, E and I: Steel, PVC and nylon controls, respectively. B,F and J: After treatment with 2 × MIC peracetic acid. C, G and K: After treatment with 2 × MIC sodium hypochlorite. D,H and L: After treatment with 2 × MIC quaternary ammonia.

FIGURE 2 – Graphs indicating the sanitizers ‘effectiveness on the *A. acidoterrestris*spores. (●) Peracetic acid, (■) Sodium hypochlorite, (▲) Quaternary ammonia.

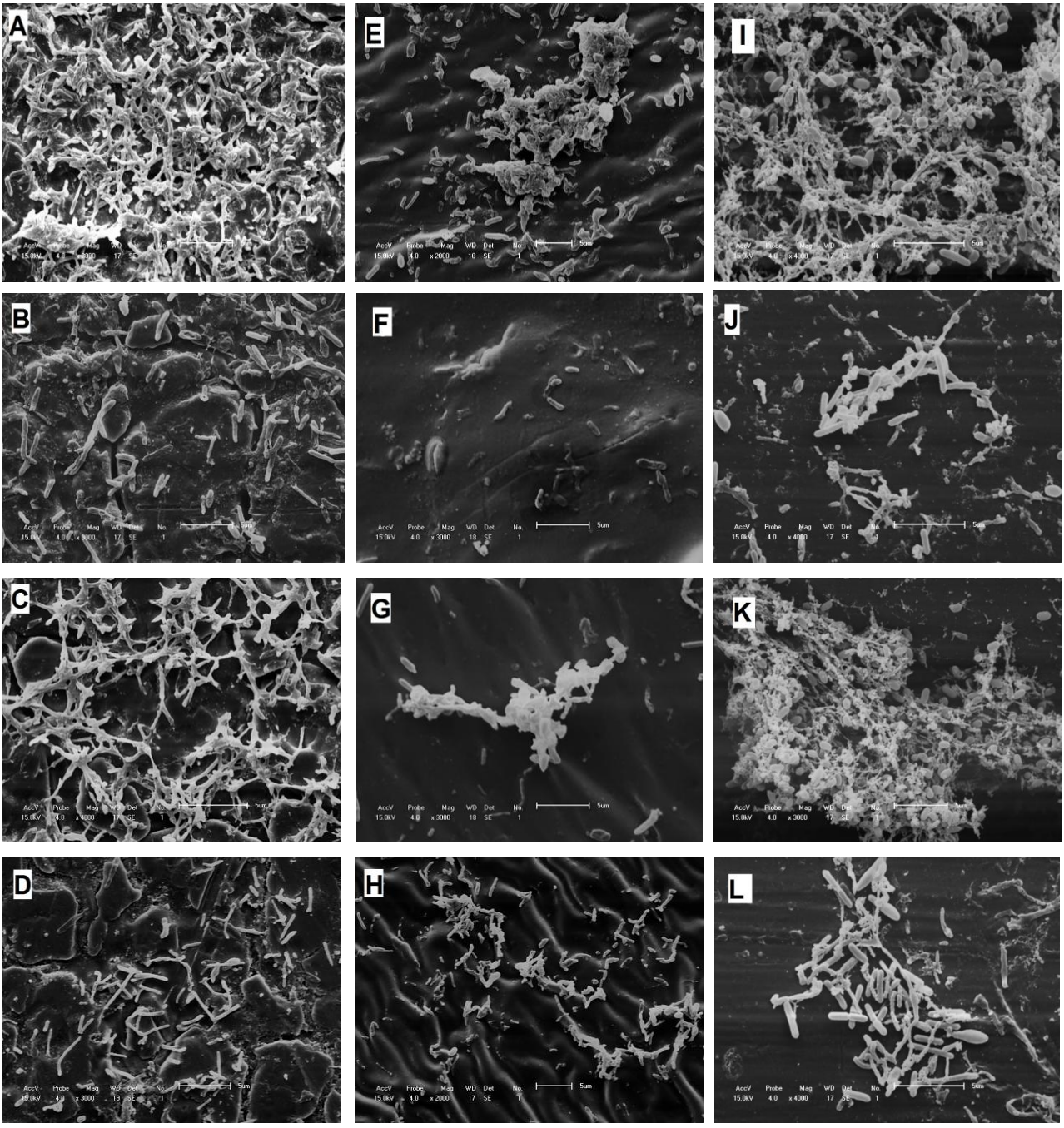
FIGURE 3 – Flow cytometry charts depicting propidium iodide staining of the vegetative cells and spores of *A. acidoterrestris* after 10 min of sanitizer treatment. A: Control vegetative cells.B: Cells treated with 500ppm peracetic acid.C: Cells treated with 1000ppm sodium hypochlorite.D: Cells treated with 15.62ppm quaternary ammonia.E: Control spores.F: Spores treated with 1000ppm peracetic acid.G: Spores treated with 1000ppm sodium hypochlorite.H: Spores treated with 40.35ppm quaternary ammonia.

TABLE 1 - Quantification of *A. acidoterrestris* on steel, PVC and nylon surfaces after treatment with peracetic acid, sodium hypochlorite and quaternary ammonia.

	Steel (log CFU/cm^2)	**PVC (log CFU/cm^2)**	**Nylon (log CFU/cm^2)**
Control	6.00±0.89^{aA}	4.85±0.52^{bA}	6.43±0.08^{aA}
MIC Peracetic Acid	4.10±0.64^{aBC}	2.65±0.27^{bCD}	4.19±0.05^{aCD}
2× MIC Peracetic Acid	3.97±0.66^{aC}	1.88±0.27^{bD}	3.8±0.04^{aD}
MIC Sodium Hypochlorite	4.97±0.15^{abB}	4.44±0.22^{bA}	5.72±0.03^{aAB}
2× MIC Sodium Hypochlorite	4.26±0.67^{abBC}	3.54±0.86^{bB}	4.86±0.33^{aBC}
MIC Quaternary Ammonia	4.41±0.13^{aBC}	3.32±0.06^{bBC}	4.76±0.02^{aC}
2× MIC Quaternary Ammonia	4.17±0.1^{aBC}	3.14±0.04^{bBC}	4.64±0.02^{aCD}

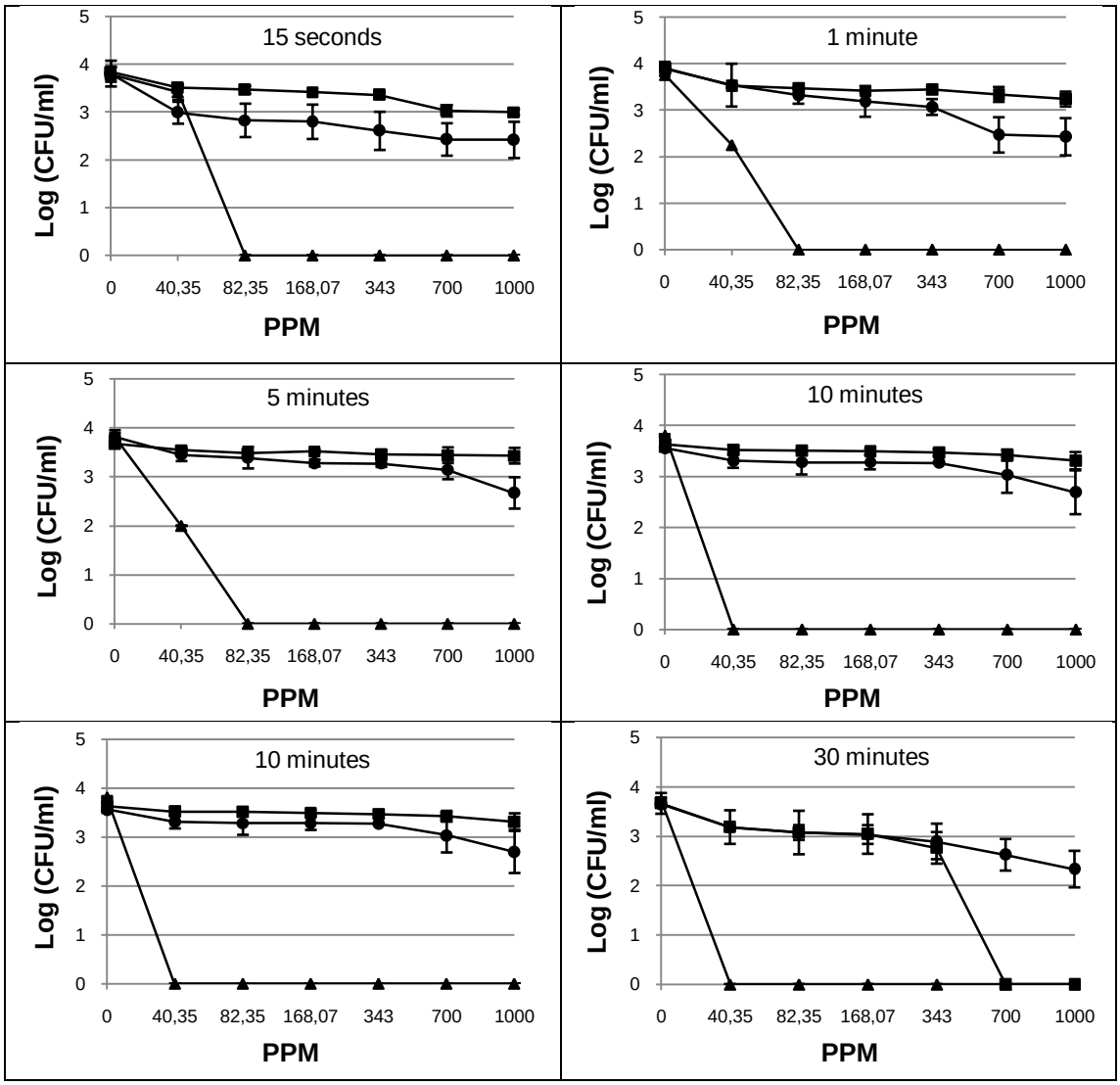
The differences between surfaces (a,b) and between treatments for each surface (A,B,C,D) annotated with the same letter are not significant ($p>0.05$).

Figure 1 –



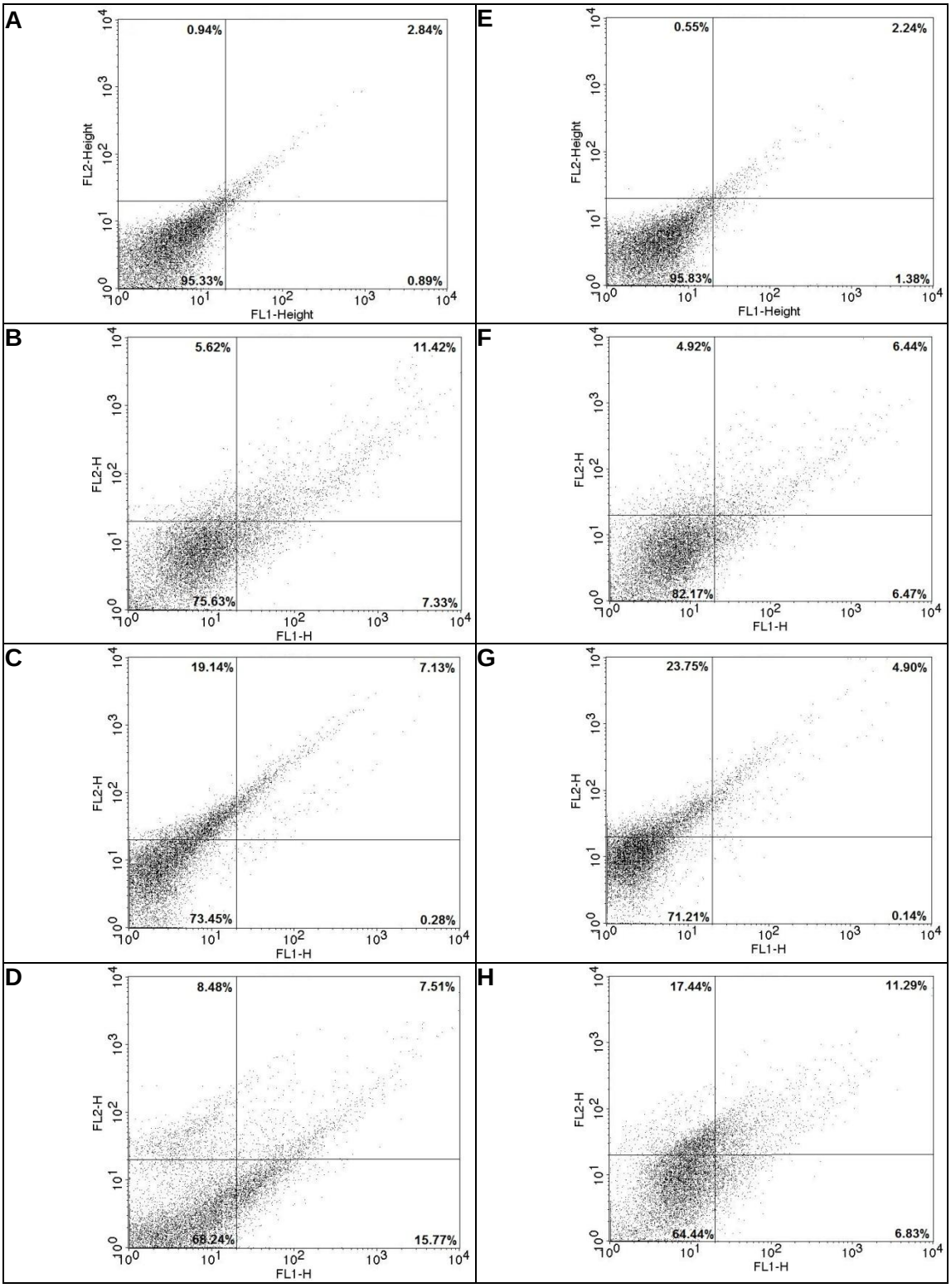
A, E and I: Steel, PVC andnylon controls, respectively. B,F and J: After treatment with 2 × MIC peracetic acid. C,G and K: After treatment with 2 × MIC sodium hypochlorite. D,H and L: After treatment with 2 × MICquaternary ammonia.

Figure 2 –



(●) Peracetic acid, (■) Sodium hypochlorite,(▲) Quaternary ammonia

Figure 3 –


A
0.94%
2.84%
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B
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FL2-H
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D
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E
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F
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23.75%
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