

## Elongated cells of *Listeria monocytogenes* in biofilms in the presence of sucrose and bacteriocin-producing *Leuconostoc mesenteroides* A11

Formação de células alongadas de *L. monocytogenes* em biofilmes na presença de sacarose e *Leuconostoc mesenteroides* A11 produtor de bacteriocina

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### Abstract

*Listeria monocytogenes* is a foodborne pathogen which may survive in biofilms and persist in food processing plants. In this study, the ability of *Leuconostoc mesenteroides* (bac<sup>+</sup> and bac<sup>-</sup>) to inhibit biofilm formation by *L. monocytogenes* ATCC 19115 was studied with stainless steel coupons immersed in BHI broth and BHI broth plus sucrose in combination with the Lactic Acid Bacteria (LAB). Adhered cells were collected with swabs and enumerated on selective agars (Oxford for listeria and MRS for leuconostoc). *Leuconostoc mesenteroides* bac<sup>+</sup> in co-culture with *L. monocytogenes* was effective to inhibit biofilm formation by listeria for up to 3 hours of incubation, but at 24 hours, biofilm was present in all conditions tested, as confirmed by observations of stainless steel coupons under Scanning Electron Microscopy (SEM). It was also observed that in the presence of *L. mesenteroides* bac<sup>+</sup> in BHI plus sucrose, a high number of elongated cells of *L. monocytogenes* was present, which may indicate an adaptation response of the pathogen to stress conditions with important implications for food safety.

**Keywords:** *L. monocytogenes*; biofilm; elongated cells; lactic acid bacteria; bacteriocins.

### Resumo

*Listeria monocytogenes* é um patógeno transmitido por alimentos que apresenta a capacidade de sobreviver em biofilmes e persistir em plantas de processamento de alimentos. O presente trabalho avaliou a habilidade de *Leuconostoc mesenteroides* (bac<sup>+</sup> e bac<sup>-</sup>) de inibir a formação de biofilme por *L. monocytogenes* ATCC 19115 em lâminas de aço inoxidável imersas em caldo BHI com ou sem sacarose em combinação com bactérias lácticas (BAL). As células aderidas nas lâminas foram coletadas com swabs e enumeradas em meios seletivos (Oxford para listeria e MRS para leuconostoc). *Leuconostoc mesenteroides* bac<sup>+</sup> foi efetivo na inibição da formação de biofilme por *L. monocytogenes* por até 3 horas de incubação. Entretanto, após 24 horas de incubação, foi verificada a formação de biofilme em todas as condições testadas, conforme evidenciado pelas imagens obtidas por meio de Microscopia Eletrônica de Varredura (MEV). Adicionalmente, foi observado um elevado número de células de *L. monocytogenes* alongadas na presença de *L. mesenteroides* bac<sup>+</sup> em BHI adicionado de sacarose, indicando uma resposta de adaptação do patógeno às condições de estresse, com importantes implicações para a segurança alimentar.

**Palavras-chave:** *L. monocytogenes*; biofilme; células alongadas; bactérias lácticas; bacteriocinas.

## 1 Introduction

*Listeria monocytogenes* is a psychrotrophic Gram-positive pathogen that tolerates relatively high-salt concentrations and a wide pH range (5.0 to 9.0). It causes invasive listeriosis, a mainly foodborne disease that is a threat for immunocompromised individuals and during pregnancy, when it may lead to central nervous systems infections and abortion. However, more recently, gastroenteritis caused by *L. monocytogenes* has also been reported (DJORDJEVIC; WIEDMANN; McLANDSBOROUGH, 2002; MEAD et al., 1999; NORWOOD; GILMOUR, 1999; SCHLECH et al., 2005).

Contamination with *L. monocytogenes* in Ready-To-Eat (RTE) foods is of special concern because it can adhere to abiotic surfaces in food processing facilities creating a cellular mass that joins nutritious residues and other microorganisms forming biofilms (COSTERTON et al., 1995; LUNDÉN; AUTIO; KORKEALA, 2002; TOMPKIN, 2002; WONG, 1998).

*L. monocytogenes* can adapt to environmental stresses commonly found during food production and this favors its persistence in biofilms, which compromises food safety since adhered cells can be resistant to sanitizers and more difficult to detect and eliminate (ADRIÃO et al., 2008; BEREKSI et al., 2002; GIOTIS; BLAIR; McDOWELL, 2007; ZAIKA; FANELLI, 2003).

Morphological changes in bacterial cells may result as response to adverse conditions including exposure to acids, high CO<sub>2</sub> concentration, high osmolarity, non optimum temperatures, and antimicrobial agents (BEREKSI et al., 2002; GIOTIS; BLAIR; McDOWELL, 2007; HAZELEGER; DALVOORDE; BEUMER, 2006; ISOM et al., 1995; JYDEGAARD-AXELSEN et al., 2005; LI et al., 2003; MINKOWSKI et al., 2001; NILSSON et al., 2000; ZAIKA; FANELLI, 2003). It has been reported that stress conditions may cause elongation of cells of several foodborne pathogens such as *Salmonella*, *Escherichia coli*, *Bacillus*,

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*Clostridium* and *L. monocytogenes* (EVERIS; BETTS, 2001; GILL; BADONI; JONES, 2007; HAZELEGER; DALVOORDE; BEUMER, 2006; JONES; GILL; McMULLEN, 2003; KIEBOOM et al., 2006).

Elongated cells may present an increased ability to adapt to subsequent stresses and can easily split up into single cells and start growing rapidly when transferred to more favourable conditions resulting in a highly contaminated food product (HAZELEGER; DALVOORDE; BEUMER, 2006; MATTICK et al., 2000; ZAIKA; FANELLI, 2003).

An alternative to prevent contamination of foods is the use of antimicrobial peptides named bacteriocins that may be active against spoilage and pathogenic bacteria such as *L. monocytogenes*. Some species of *Leuconostoc* can produce bacteriocins and are commonly used as starters in dairy fermentation (DE MARTINIS; FREITAS; SANTAROSA, 2003; KANG et al., 2007). *Leuconostoc* spp. comprises a group of heterofermentative LAB and some isolates synthesize extracellular polysaccharides (such as dextran from sucrose), which potentially interfere with biofilm formation (KANG et al., 2007).

In this paper, the ability of biofilm formation by *L. monocytogenes* on stainless steel coupons was evaluated in the presence of bacteriocin-producing and non bacteriocin-producing isolates of *L. mesenteroides*.

## 2 Materials and methods

### 2.1 Bacterial cultures

The bacterial cultures used were: *Listeria monocytogenes* ATCC 19115, *Leuconostoc mesenteroides* A11 (bac<sup>+</sup>), a bacteriocin-producing chicken isolate (DE MARTINIS et al., 2001), and the non bacteriocin-producing meat isolate *Leuconostoc mesenteroides* A13 (bac<sup>-</sup>), kindly donated by Prof. Mariza Landgraf (Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Brazil). All cultures were kept in broths added of 20 mL.100 mL<sup>-1</sup> of glycerol at -70 °C: Brain Heart Infusion (BHI) for listeria and de Man Rogosa Sharpe (MRS) for leuconostoc (both from Oxoid, UK). The working cultures were prepared by inoculation of 1ml of stock suspension in 100 mL of suitable broth, followed by incubation for 24 hours at 37 °C (listeria) and 25 °C (leuconostoc).

### 2.2 Stainless steel coupons

Stainless steel coupons (AISI 340) of 15 cm<sup>2</sup> (7.5 × 2.0 × 0.2 cm) were washed and cleaned as previously described by Minei et al. (2008). For the experiments, up to 6 coupons were clamped vertically to a sterile stainless steel circular rack (10 cm of diameter).

### 2.3 Evaluation of biofilm formation on stainless steel coupons by pure culture of *L. monocytogenes*

One set of rack and coupons was immersed in 100 mL of BHI broth containing ca. 10<sup>5</sup> CFU.mL<sup>-1</sup> of an overnight culture of *L. monocytogenes* and incubated at 37 °C for up to 48 hours. The coupons were removed after 3, 24, and 48 hours and rinsed

with 20 mL of Phosphate Buffer Saline (PBS) to remove non-adherent cells. The adherent cells were dislodged from each slide by rubbing with sterile cotton swab approximately 100 times. Individual swabs were transferred to tubes containing 10 mL of PBS and vortexed to suspend the cells in PBS. Serial decimal dilutions were prepared and surface plated on Trypticase Soy agar supplemented with 0.6 g.100 mL<sup>-1</sup> of yeast extract (TSAYE, Oxoid - UK). The plates were incubated at 37 °C for 24 hours to estimate *L. monocytogenes* population (CHAE; SCHRAFT, 2000; MARSH; LUO; WANG, 2003).

### 2.4 Evaluation of biofilm formation on stainless steel coupons by a pure culture of *L. mesenteroides*

One set of rack and coupons was immersed in 100 mL of BHI broth inoculated with 500 µl of overnight cultures of either *L. mesenteroides* bac<sup>+</sup> or bac<sup>-</sup>. Broths were incubated at 30 °C for up to 48 hours and the coupons were removed after 3, 24, and 48 hours and rinsed with 20 mL of PBS to remove non-adhered cells. The adherent cells were removed with swabs from the slides and plated as described above, but using MRS agar. The plates were incubated at 25 °C for 48 hours to estimate populations of leuconostocs (CHAE; SCHRAFT, 2000; MARSH; LUO; WANG, 2003).

### 2.5 Influence of co-culture with *L. mesenteroides* bac<sup>+</sup> and bac<sup>-</sup> on *L. monocytogenes* biofilm formation

Working cultures of *L. monocytogenes*, *L. mesenteroides* bac<sup>+</sup> and bac<sup>-</sup> were prepared as described in item 2.1 and used to inoculate (500 µl) BHI broth (100 mL) containing a set with rack and coupons under the following conditions: a) *L. monocytogenes* plus *L. mesenteroides* bac<sup>+</sup>, b) *L. monocytogenes* plus *L. mesenteroides* bac<sup>-</sup>. Incubation was performed at 25 °C for up to 48 hours, with removal of coupons after 3, 24, and 48 hours to check for bacterial adherence. The slides were swabbed and serial decimal dilutions were prepared and surface plated (100 µL) on MRS agar (leuconostoc) and Oxford agar (listeria). Incubation was performed respectively at 25 and 37 °C for 24 hours. Additionally, the same experimental design was repeated using BHI broth added of 2 g.100 mL<sup>-1</sup> sucrose (Synth, Brazil).

### 2.6 Scanning Electron Microscope (SEM)

Round stainless steel coupons (1.2 cm diameter) were prepared to be analyzed by SEM, and images were obtained at 24 hours of incubation as described by Minei et al. (2008).

### 2.7 Statistical analysis

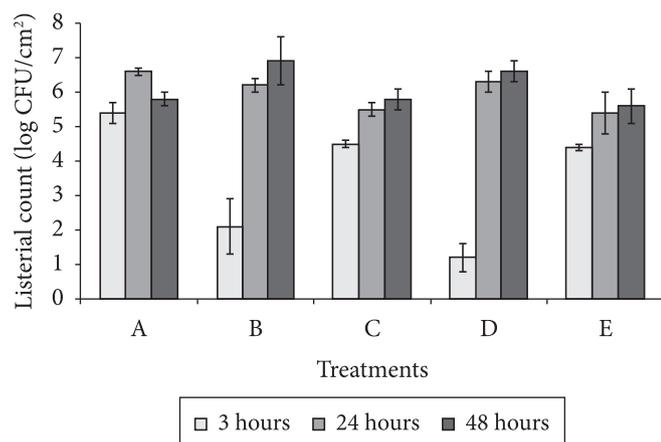
The data presented are averages of three independent replicates and mean ± standard deviation. To evaluate the differences among the treatments applied, one-way ANOVA with a significance level of p < 0.05 was used. When statistically significance difference among treatments was found, the Student's Newman Keuls test was applied with a significance level of p < 0.05. Analyses were performed using the software package SigmaStat 3.11 (SYSTAT, 2004).

### 3 Results and discussion

It was previously demonstrated that dextrans produced by *Leuconostoc* spp. inhibited growth *in vitro* and formation of oral biofilms by *Streptococcus mutans* (KANG et al., 2007), and that the food isolate *L. mesenteroides* A11 presented antimicrobial activity towards *L. monocytogenes* (DE MARTINIS; FREITAS; SANTAROSA, 2003; MARTINEZ; DE MARTINIS, 2006). However, there are no reports in the literature on biofilm formation by *L. monocytogenes* in co-culture with *L. mesenteroides*.

For the present study, biofilm formation was considered when at least  $10^3$  cells were adhered per  $\text{cm}^2$  (WIRTANEN; HUSMARK; MATTILA-SANDHOLM, 1996). *L. monocytogenes* alone formed biofilm after 3 hours of incubation ( $5.4 \log \text{CFU.cm}^{-2}$ ) with increasing number of adhered cells at 24 hours ( $6.6 \log \text{CFU.cm}^{-2}$ ), followed by a decrease at 48 hours of incubation ( $5.8 \log \text{CFU.cm}^{-2}$ ) (Figure 1). *L. mesenteroides*  $\text{bac}^+$  did not adhere to the stainless steel coupons, whereas *L. mesenteroides*  $\text{bac}^-$  formed biofilm after 24 hours and presented a maximum number of adhered cells after 48 hours of incubation ( $5.9 \log \text{CFU.cm}^{-2}$ ) (Figure 2).

When *L. mesenteroides*  $\text{bac}^+$  was co-cultivate with *L. monocytogenes*, it formed biofilm after 48 hours with  $4.3 \log \text{CFU.cm}^{-2}$  (Figure 2). Moreover, it is important to note that co-culture with *L. mesenteroides*  $\text{bac}^+$  caused a reduction of up to 3.2 log cycles in adhered population of *L. monocytogenes* (Figure 1) in the first 3 hours of incubation ( $p < 0.001$ ). After 24 hours of incubation, the number of adhered cells was still lower ( $6.1 \log \text{CFU.cm}^{-2}$ ) than *L. monocytogenes* in pure culture ( $6.6 \log \text{CFU.cm}^{-2}$ ) with statistical significance ( $p < 0.050$ ). However, it was observed a significant ( $p = 0.025$ ) increase of the number of *L. monocytogenes* cells adhered to the stainless steel surface within 48 hours of incubation in the presence of *L. mesenteroides*  $\text{bac}^+$ .



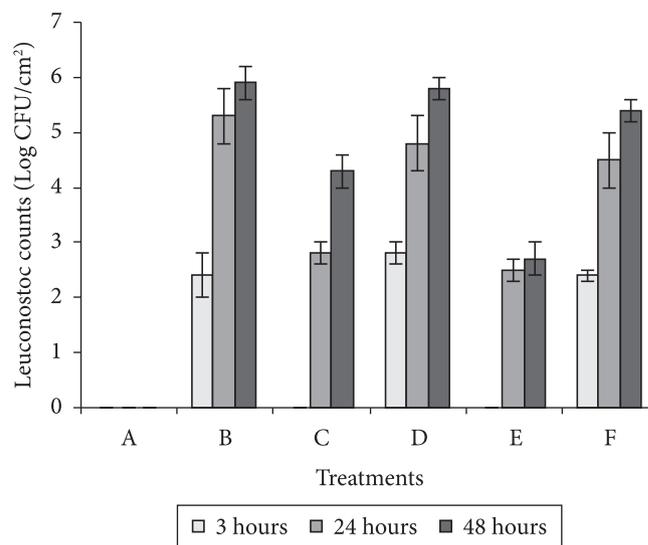
**Figure 1.** Biofilm formation by *L. monocytogenes* ATCC 19115 growing at 30 °C in BHI broth after 24 hours of incubation on stainless steel coupons: a) pure culture; b) co-culture with *L. mesenteroides* A11 ( $\text{bac}^+$ ) and c) co-culture with *L. mesenteroides* A13 ( $\text{bac}^-$ ). *L. monocytogenes* ATCC 19115 growing at 30 °C in BHI broth added of sucrose ( $2 \text{ g.}100 \text{ mL}^{-1}$ ) after 24 hours of incubation on stainless steel coupons: d) co-culture with *L. mesenteroides* A11 ( $\text{bac}^+$ ); and e) co-culture with *L. mesenteroides* A13 ( $\text{bac}^-$ ). Error bars indicate standard deviation from three independent experiments. Dotted line indicates threshold value ( $10^3$  cells) considered as biofilm formation.

Biofilm formation by *L. mesenteroides*  $\text{bac}^-$  in co-culture with *L. monocytogenes* was observed after 24 hours of incubation with  $5.8 \log \text{CFU.cm}^{-2}$  of adhered cells at 48 hours (Figure 2). Influence on biofilm formation by *L. monocytogenes* was not significant in the presence of *L. mesenteroides*  $\text{bac}^-$  (Figure 1). For plate count results, there were not statistically significant differences among co-culture experiments carried out with BHI broth or BHI broth with sucrose (Figures 1 and 2).

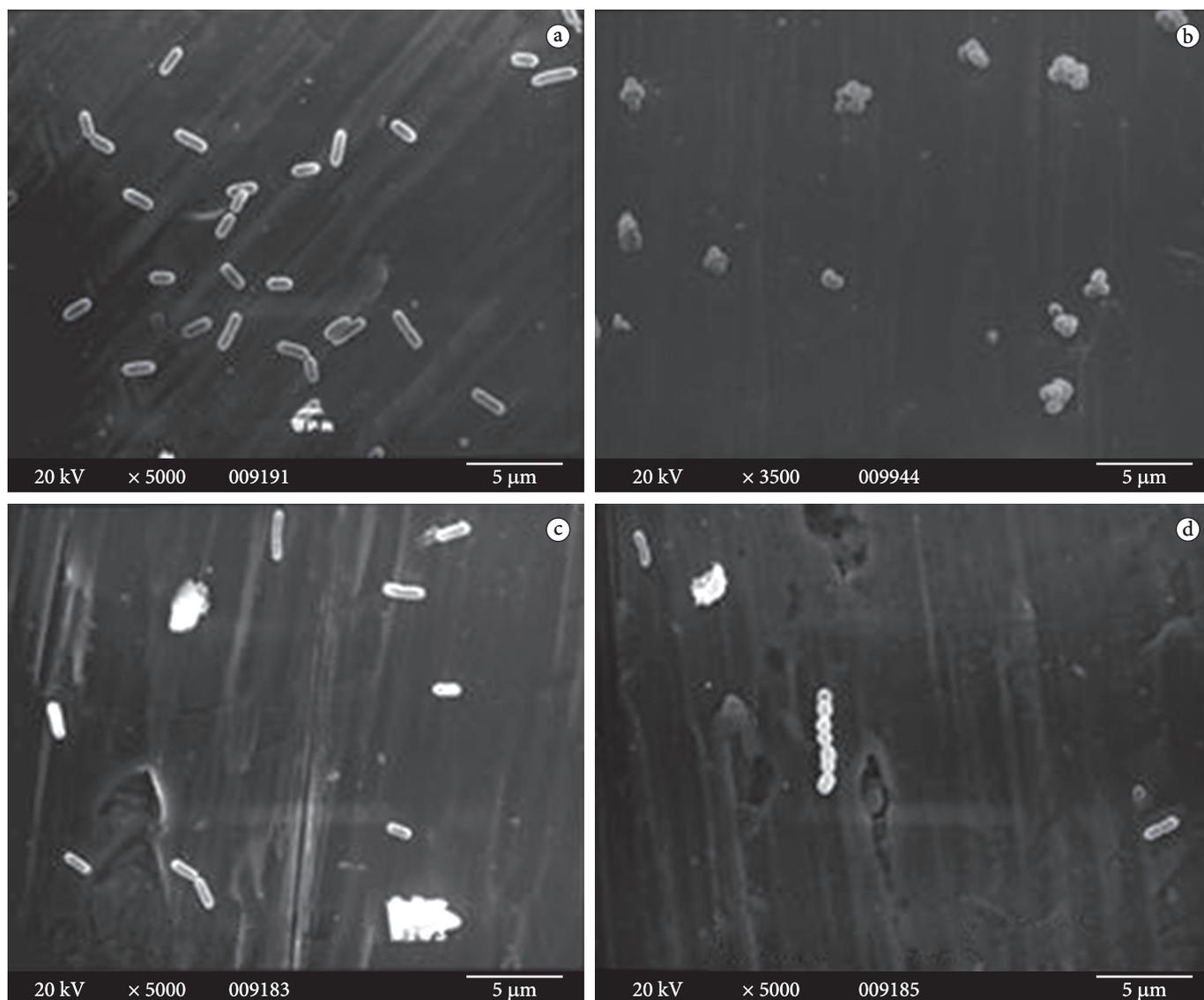
Minei et al. (2008) evaluated the effect of LAB strains in the ability of biofilm formation by *L. monocytogenes* and revealed that when *L. monocytogenes* was co-cultured with *E. faecium*  $\text{bac}^-$ , in the early hours of incubation, the number of adhered *L. monocytogenes* cells was 2.5 log lower compared with that in the control, but after 6 hours of incubation biofilm it was detected again. However, the co-culture of *E. faecium*  $\text{bac}^+$  and *L. monocytogenes* did not allow biofilm formation.

According to Carpentier and Chassaing (2004), microorganisms present in food processing premises can either enhance or inhibit *L. monocytogenes* colonization on inert surfaces depending on the diversity and biochemical activity of accompanying microflora.

Several authors have applied SEM to analyze biofilms on abiotic surfaces (KALMOKOFF et al., 2001; MARSH; LUO; WANG, 2003; MOLTZ; MARTIN, 2005; MINEI et al., 2008). In this study, images captured by SEM showed that *L. monocytogenes* formed biofilm (ca.  $10^5$ - $10^6 \text{ CFU.cm}^{-2}$ ) within 24 hours of incubation on stainless steel coupons in all tested conditions (Figures 3 and 4), confirming data obtained



**Figure 2.** Biofilm formation by *Leuconostoc* A11 ( $\text{bac}^+$ ) after 24 hours of incubation at 30 °C on stainless steel coupons in BHI broth: a) pure culture; c) co-culture with *L. monocytogenes* ATCC 19115, and e) co-culture with *L. monocytogenes* ATCC 19115 in BHI broth added of sucrose ( $2 \text{ g.}100 \text{ mL}^{-1}$ ). Biofilm formation by *L. mesenteroides* A13 ( $\text{bac}^-$ ) after 24 hours of incubation on stainless steel coupons: b) pure culture; d) co-culture with *L. monocytogenes* ATCC 19115, and f) co-culture with *L. monocytogenes* ATCC 19115 in BHI broth added of sucrose ( $2 \text{ g.}100 \text{ mL}^{-1}$ ). Error bars indicate standard deviation from three independent experiments. Dotted line indicates threshold value ( $10^3$  cells) considered as biofilm formation.



**Figure 3.** Scanning electron micrographics showing biofilm formation by a) pure culture of *L. monocytogenes* ATCC 19115; b) pure culture of *L. mesenteroides* A13 (bac<sup>-</sup>); c) co-culture of *L. monocytogenes* ATCC 19115 plus *L. mesenteroides* A11 (bac<sup>+</sup>); and d) co-culture of *L. monocytogenes* ATCC 19115 plus *L. mesenteroides* A13 (bac<sup>-</sup>) on stainless steel coupons after 24 hours of incubation at 30 °C in BHI broth.

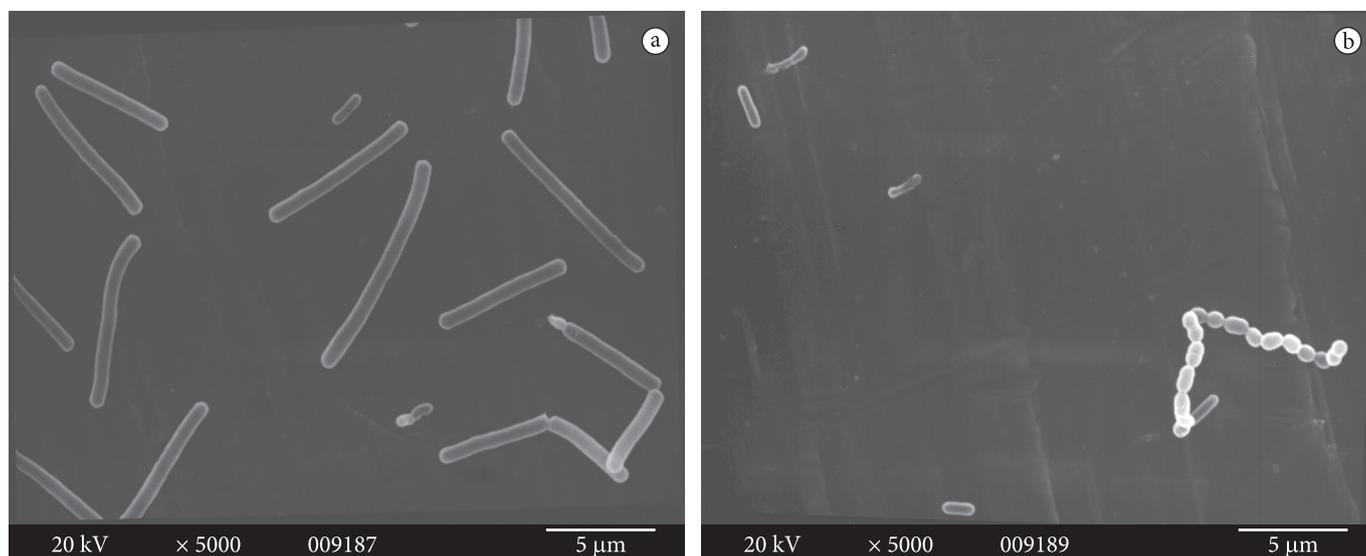
by the plate count method. *L. monocytogenes* in pure culture or co-cultivated with *L. mesenteroides* bac<sup>+</sup> in BHI broth without the addition of sucrose exhibited normal cell length of ca. 1.6 μm (Figure 3a, c and d). Biofilm formation was observed for *L. mesenteroides* bac<sup>-</sup> alone (Figure 3b), but not for *L. mesenteroides* bac<sup>+</sup> (data not shown).

Interestingly, elongated cells of *L. monocytogenes* (longer than 6.6 μm) were observed in biofilm in the presence of *L. mesenteroides* bac<sup>+</sup> in BHI broth with sucrose (Figure 4a). Similar results were found by Kieboom et al. (2006) in a study on morphological changes and cell viability of *Salmonella* exposed to reduced water activity and different temperatures. Sucrose may be implicated with the formation of elongated cells since no morphological change was observed when *L. monocytogenes* was co-cultivated with *L. mesenteroides* bac<sup>+</sup> without sucrose (Figure 3c).

Despite diverse studies on the effects of stress conditions in the morphology and viability of *L. monocytogenes* (HAZELEGER; DALVOORDE; BEUMER, 2006; ZAIKA; FANELLI, 2003), this is the first report on the effect of sucrose plus bacteriocin-producing lactic acid bacterium in the altered morphology of *L. monocytogenes*. According to Hazeleger, Dal Voorde and Beumer (2006), elongated cells of *Listeria* and *Salmonella* stained with DAPI (a nucleic acid stain) presented cell septa indicating that those cells were able to split up rapidly in single cells under more favorable conditions.

#### 4 Conclusions

*L. mesenteroides* bac<sup>+</sup> was more effective than *L. mesenteroides* bac<sup>-</sup> in delaying biofilm formation by *L. monocytogenes* on stainless steel surface indicating its potential application to improve food safety. Sucrose and bacteriocins may influence the



**Figure 4.** Scanning electron micrographics showing biofilm formation by a) co-culture of *L. monocytogenes* ATCC 19115 (elongated cells) plus *L. mesenteroides* A11 (bac<sup>+</sup>) and b) co-culture of *L. monocytogenes* ATCC 19115, plus *L. mesenteroides* A13 (bac<sup>-</sup>) on stainless steel coupons after 24 hours of incubation at 30 °C in BHI broth containing 2 g.100 mL<sup>-1</sup> of sucrose.

behavior of *L. monocytogenes* in food systems since the elongated cells of *L. monocytogenes* were observed and may indicate an adaptation mechanism to stress conditions.

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