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Role of Lactic Acid Bacteria as a Bio-sanitizer to Prevent Attachment of Listeria Monocytogenes on Deli Slicer Contact Surfaces, and the Influence of Listeria Innocua on the Attachment of L. Monocytogenes on Selected Materials Located in the Food-processing Environment

Jean Baptiste Ndahetuye
University of Arkansas, Fayetteville

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ROLE OF LACTIC ACID BACTERIA AS A BIO-SANITIZER TO PREVENT ATTACHMENT OF LISTERIA MONOCYTOGENES ON DELI SLICER CONTACT SURFACES, AND THE INFLUENCE OF LISTERIA INNOCUA ON THE ATTACHMENT OF L. MONOCYTOGENES ON SELECTED MATERIALS LOCATED IN THE FOOD-PROCESSING ENVIRONMENT

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A thesis submitted in partial fulfillment

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By

Jean Baptiste Ndahetuye

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ABSTRACT

Listeria monocytogenes is an important foodborne pathogen that continues to be a serious problem for the food industry. This pathogen contaminates food primarily during post-harvest in the food-processing environment. Its ecology in the food-processing environment is not well understood but previous research has demonstrated the ability of *L. monocytogenes* to survive on food contact surfaces after cleaning and disinfection. The current study explored the attachment of three lactic acid bacteria (LAB) strains, *Lactobacillus animalis* , *Lb. amylovorus* and *Pediococcus acidilactici*, their combination in a cocktail, and their influence to prevent the attachment of *L. monocytogenes* at room temperature by examining their cell surface hydrophobicity, total carbohydrates production and adherence capability to stainless steel coupons made from a deli slicer. Subsequently, the study evaluated the influence of *L. innocua* on the attachment of *L. monocytogenes* on stainless steel, and on aluminum surfaces. The hydrophobicity tests were performed according to microbial adhesion to solvent (MATS). Extracellular carbohydrates were quantified using a colorimetric method. Based on these tests, *Lb. animalis* exhibited the greatest hydrophobicity (26.3 %) and its adherence increased sharply from 24 to 72 hr, whereas *Lb. amylovorus* yielded the lowest hydrophobicity 3.86 % and was only weakly adherent. *P. acidilactici* with a hydrophobicity of 10.1 % adhered strongly. The attached LAB strains produced significantly ($P < 0.05$) higher total carbohydrates than their planktonic counterparts, which is an important characteristic for attachment. Three separate conditions, LAB first, LAB and *L. monocytogenes* concurrently and *L. monocytogenes* inoculated first then LAB, were simulated to evaluate the ability of the LAB cocktail (10^8 CFU/ml) to competitively exclude *L. monocytogenes* (10^3 CFU/ml) on the surface of the stainless coupons. In all the three comparisons, the LAB cocktail was able to reduce the attachment of *L. monocytogenes* significantly ($P < 0.05$). Thus, the LAB cocktail indicated

attachment on stainless steel and bacteriostatic activity against *L. monocytogenes* attached on stainless steel. These properties may be used in manufacturing a bio-sanitizer geared to prevent the formation *L. monocytogenes* biofilm on food contact surfaces. Preliminary data showed *L. innocua* had a positive effect on the attachment of *L. monocytogenes* on stainless steel or aluminum surfaces.

This thesis is approved for recommendation
to the Graduate council.

Thesis Director:

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Dr. Philip G. Crandall

_____

Dr. Navam S. Hettiarachchy

_____

Dr. Frank Millet

_____

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I.   INTRODUCTION

Listeria monocytogenes is an important foodborne pathogen that is widely distributed in nature, and the pathogen is responsible for life-threatening illnesses such as meningitis, and meningo-encephalitis (Cossart and Toledo-Arana, 2010; Garcia-Almendarez et al., 2008). It has the ability to attach and form biofilms on several types of materials located in the food-processing environment such as stainless steel, aluminum, conveyer belts made in polyester and others (Beresford et al., 2001; Somers and Wong, 2004). Attachment and biofilm formation enhances the persistence and resistance of *Listeria* to cleaning and disinfection (Simoes et al., 2010). Many foodborne outbreaks have implicated *L. monocytogenes* as a cross-contaminant from the food-processing environment (Farber and Peterkin, 1991). Such outbreaks generally result in high hospitalization or mortality rates along with immense food product recalls, which cause heavy financial burdens to the health care systems and the suspected food manufacturer (Scallan et al., 2011; Scharf, 2010).

Ready to eat (RTE) foods have been implicated in *L. monocytogenes* outbreaks, and studies have demonstrated that the pathogen may transfer from a contaminated deli slicer to food (Lin et al., 2006). Availability of nutrients, humidity and repeated use of the deli slicer increase the likelihood of *L. monocytogenes* attachment to its surface (Chmielewski and Frank, 2003). In addition, deli slicers are widely used in many retail establishments, which highlights the public health risk associated with cross contamination from this pathogen via the deli slicer. Although the risk associated with *L. monocytogenes* is well known, understanding of the pathogen ecology and survival within the foo

d-processing environment is limited. Since *L. monocytogenes* is widely distributed in nature, and may access processing environment, preventing adhesion of the pathogen to a food contact surface appears a better control strategy for food safety.

Interaction of several microorganisms attached to surfaces may interfere or promote *L. monocytogenes* biofilm formation and development. Norwood and Gilmour (2001) observed the decrease of *L. monocytogenes* cell numbers attached to stainless steel at 4, 18, and 30° C in the presence of *Staphylococcus xylosus* and *Pseudomonas fragi*. The decrease was attributed to the competition for nutrients and production of antagonistic compounds by *S. xylosus*. However, a separate study done by Sasahara and Zottola (1993) observed that attachment of *L. monocytogenes* in mixed-cultures was enhanced by the exopolymer produced by *P. fragi* as compared to the *L. monocytogenes* in pure culture. The non-pathogenic *L. innocua* is abundant in the environment and has been isolated in the same niches with *L. monocytogenes* such as ground water and surface water samples, or in fecal samples from the soil (Luppi et al., 1988; Weis and Seeliger, 1975; Wilshimer, 1968). Investigating the influence of *L. innocua* on the survival of *L. monocytogenes* in niches in the food-processing environment would enhance our understanding of the ecology of *L. monocytogenes* in the food-processing environment.

Consumer demand for foods processed with minimum use of chemical preservatives has made lactic acid bacteria (LAB) a desirable approach to control *L. monocytogenes* in foods or in the processing environment. LAB have a proven record of controlling food spoilage and pathogens in food and in the environment (Bernardi et al., 2011; Brown et al., 2011). LAB are able to affect microbial ecology through production of organic acids, hydrogen peroxide or bacteriocins that suppress the growth and multiplication of competitive microorganisms (Jay, 1982).

Historically, LAB have had a record of safe applications in food and food products. The use of LAB as a starter cultures in making sausages is common and recently LAB have been used as protective cultures because of their ability to produce antimicrobial metabolites and to compete for nutrients with pathogenic or spoilage microorganisms. Another beneficial property of LAB is a continuous production of antimicrobial metabolites under refrigeration conditions without growth of the LAB (FDA, 2005). For this reason some LAB strains have found applications in the control of *L. monocytogenes* in meat products (Amezquita and Brashears, 2002). Undoubtedly, LAB appear to be the best candidate for further exploration of competitive exclusion of *L. monocytogenes* on deli slicer food contact surfaces. The current scientific study characterized the attachment of three LAB strains, their combination in a cocktail and *L. monocytogenes* on stainless steel surfaces. The competitive exclusion of *L. monocytogenes* by LAB cocktails on stainless steel was also evaluated. Subsequently, the effect of *L. innocua* on the attachment of *L. monocytogenes* on stainless steel and aluminum was evaluated. The cocktails of LAB strains were able to attach to stainless steel and reduced significantly ($P < 0.05$) the attachment of *L. monocytogenes*. Additionally, preliminary data demonstrated that *L. innocua* might affect the attachment of *L. monocytogenes* on tested surfaces, and real-time polymerase chain reactions tests would confirm the results.

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II. LITERATURE REVIEW

Foodborne pathogens cause significant problems in the food supply chain worldwide, and the illness and death caused by eating contaminated food are of a great concern for the public health officials. Globalization of food marketing and distribution could conceivably lead to contaminated food products from a single source that result in illness in many people in different countries (Kuchenmüller et al., 2009). Despite the efforts in controlling food contamination and development in detection methods, the number of foodborne diseases has been increasing (WHO, 2007). It is estimated that approximately 30 % of the emerging diseases in past 60 years are transmitted to humans though food (Kuchenmüller et al., 2009).

Foodborne pathogens also pose serious problems in the United States, where they cause many illnesses and deaths. It is estimated that foodborne diseases cause 9.4 million of illness each year. It is also estimated that out of the 9.4 million, 3.6 million (39%) are caused by bacteria (Scallan et al., 2011). The financial burden is reflected through the healthcare costs and the human loss resultant from contradicting a foodborne pathogen (Scharff, 2010). For instance, *L. monocytogenes*, one of the foodborne pathogens with high hospitalization and mortality rates; 94.0 and 15.9 %, respectively, causes 1591 illnesses annually, which costs US healthcare system around $ 2.7 billion (Scallan et al., 2011; Scharff, 2010).

The food industry has intensified efforts to control foodborne pathogens through research and development programs; however, outbreaks linked to consumption of contaminated foods persist. Food-processing environments are one of the major sources of food contamination (Bremer et al., 2001). Research in elucidating the ecology and reservoirs of foodborne pathogens and developing novel cleaning technologies is still needed. Preventing contamination of surfaces

in ready- to- eat (RTE) food establishments is important because RTE foods are eaten without further heat treatment (Gallagher, 2003). Demand of RTE foods have increased due to consumers' preference for convenience foods throughout the world, the number of susceptible to listeriosis persons has also increased in North America and Europe, which makes prevention of the pathogen a research priority (Walter, 2000). RTE foods that have been implicated in *L. monocytogenes* outbreaks include hot dogs, deli meats or Mexican-style cheese (CDC, 2011). RTE foods generally undergo lethality treatment; however, following processing, *L. monocytogenes* on food-contact surfaces of equipment can contaminate RTE foods in a process known as post-processing contamination (USDA-FSIS, 2003). Inhibition of *L. monocytogenes* access to the food-processing environment is challenging because of the ubiquitous nature of the pathogen, and its ability to attach to and form biofilms on materials such as stainless steel, and aluminum that are found in the food-processing environment (Beresford et al., 2001; Tompkin, 2002; Zhao et al., 2004).

L. monocytogenes history

Murray was the first scientist to describe the Gram- positive *L. monocytogenes* as an organism that caused infection in laboratory rabbits in 1926. He named the organism *Bacterium monocytogenes* (Murray et al., 1926). One year later, Pirie (1927) isolated a closely related organism that caused epizootics in veld rodents from South Africa. Eventually the name of *L. monocytogenes* was derived from the combined work both scientists. The pathogen continued to cause infections, and in 1929, Nyfeldt, (1929) reported the first infection of *L. monocytogenes* in a human. He identified *L. monocytogenes* as a cause of mononucleosis–like infection in blood culture from patients. In addition, Burn (1933) proved *L. monocytogenes* to be the cause of sepsis in perinatals and meningitis in adults (Gray and Killinger, 1966). Several instances of listeriosis

in animals were identified, but it was in the 1950's when listeriosis was recognized as a serious illness of humans, especially among immune-compromised. Extensive literature reviews about listeriosis infections in infants and adults have been published (Louria et al., 1966; Walter, 2000).

Research on the pathogen continued to evolve, new *Listeria* infection patterns were determined. Nieman and Lorber (1980) reported that in addition to infants and elderly, people with renal transplants were also the target of listeriosis infection. Listeriosis was causing more deaths in people with conditions such as cancer or who were immunosuppressed status (Nieman and Lorber, 1980). In 1980, the pathogen caused a large outbreak characterized by high perinatal mortality in Canada (Schlech et al., 1983). The vehicle of the pathogen was coleslaw, the first time that *L. monocytogenes* was recognized as a foodborne pathogen (Schlech et al., 1983). During the period from 1980 until 2000 outbreaks occurred in which RTE foods including hot dogs Mexican-style cheese and delicatessen turkey were the major vehicle of the pathogen (Swaminathan and Gerner-Smidt, 2007). For example, a multistate Listeria outbreak in 1985 resulted in 48 deaths, with Mexican-style cheese identified as the vehicle (Swaminathan and Gerner-Smidt, 2007). Another deadly listeriosis outbreak in France was linked to pork tongue in the jelly, where 85 deaths were reported (Swaminathan and Gerner-Smidt, 2007). The most recent outbreak occurred in the U.S 2011, a total of 148 persons were infected and 13 deaths and one miscarriage were reported. *Listeria* was traced from the equipment where cantaloupes, which were the vehicle of the pathogen, were handled (CDC, 2011)

L. monocytogenes **taxonomy**

The ability of *L. monocytogenes* to survive under refrigeration and form biofilm on materials typically used in environments where RTE foods are processed substantially elevates the public health risk associated with *L. monocytogenes* (Blackman and Frank, 1996, Sergelidis, et al. 1997)

This organism was classified into the genus of Listeria mainly because of the morphological characteristics this Gram-positive rod. The genus *Listeri*a has low G+C content ranging between 36 and 42 %, lack of mycolic acids and presence of lipoteichoic acids. DNA homology and 16s rRNA sequencing were used to determine *Listeria* species. Results demonstrated *Listeria* to have 6 species, two of which are pathogenic: *L. monocytogenes* and *L. ivanovii* and 4 are non-pathogenic: *L. innocua*, *L. welshimeri* and *L. seeligeri*, *L. grayi*. Molecular tests classified a seventh species, *L. murrayi*, as a subspecies of *L. grayi* (Recount, 1988; Jammie and Stephan, 2006). Recently *L. rocourtiae* sp. Nov. and *L. marthii* sp. nov. species were discovered and added to the genus *Listeria. L. rocourtiae* sp. Nov was isolated from pre-cut lettuce, and comparison of the gene sequence and gene content revealed that it is affiliated with genus *Listeria*. *L. marthii* sp. nov. was isolated from soil, standing water and flowing water samples from the natural environment in the Finger Lakes National Forest, New York, USA (Graves et al., 2010). Likewise, whole genome DNA–DNA hybridization and 16S rRNA gene sequence tests demonstrated the new species to belong to *Listeria* genus (Leclercq et al., 2010). *Listeria* has also been divided into serotypes on the basis of serological reactions of listerial somatic (O factor) and flagellar (H factor) antigens with specific antisera. Upon combination of O and H antigen tests, *L. monocytogenes* could be divided into serotypes 1/2 a, 1/2 b, 3 b, 4 a, 4 b, 4 c and 6 b (Liu, 2008). Serotypes 4 b and 1/2 a were the most implicated in listeriosis outbreaks in the past decade (Swaminathan and Gerner-Smidt, 2007) additionally,

molecular subtyping methods have been used concomitantly with serotyping methods to provide more epidemiologic and phylogenitical meaningful data. Molecular typing methods including multiple locus enzyme electrophoresis (MLEE), ribotyping and restriction fragment length polymorphism (RFLP)-based schemes, pulsed field gel electrophoresis (PFGE), polymerase chain reaction (PCR)-based subtyping and others have been used to divide *L. monocytogenes* into three lineages, Lineage I, II, and III. Lineage I also denoted genomic division II comprises serotypes 1/2 b, 3 b, and 4 b. Lineage II corresponding to the genomic division I includes 1/2 a, 3 a, 1/2 c and 3 c, and lineage III includes serotypes 4 a and 4 c (Wiedmann et al., 1997; Ward et al., 2004; Liu, 2008).

Characteristics of *L. monocytogenes*

L. monocytogenes is a Gram-positive rod-shaped bacterium. The rods have parallel sides and their sizes are 1 to 2 μm by 0.5 μm. Colonies of *L. monocytogenes* are round, translucent and fine in texture (Gray and Killinger, 1966). *L. monocytogenes* possesses a peritrichous flagella and exhibit tumbling motility at 20 to 25° C. *L. monocytogenes* is facultatively anaerobic, catalase positive and oxidative negative (Jemmi and Stephan, 2006). They are characterized by β hemolysis of red blood cells causing clear zones around colonies on blood agar. *L. monocytogenes* prefers a neutral pH growing environment; however, the organism can survive at a pH of 4. 5. Although the temperature range of 3° C to 45° C supports the growth of *L. monocytogenes*, its growth peaks at 37° C after 16 to 18 hours of incubation whereas growth is observed after 5 to 8 days under refrigeration conditions (Farber and Peterkin, 1991). Avery and Byrne (1959) noticed that cultures of *L. monocytogenes* at 4° C are more pathogenic than those growing at 37°C.

L. monocytogenes **in the food-processing environment.**

L. monocytogenes is widely distributed in the natural environment. It has been isolated from water and sludge (Luppi et al., 1988), soil (Weis and Seeliger, 1975) and plants and vegetation (Wilshimer, 1968). Animal farms, especially cattle operations, are also reservoirs of *L. monocytogenes* including subtypes able to cause listeriosis infection in humans. The organisms at the farms can be transmitted to food- processing plants through contaminated farm products (Nightingale et al., 2004). The ubiquity of *L. monocytogenes* is one of the reasons why it has been challenging to completely prevent or eliminate its access to food processing environment.

Raw materials entering the food-processing environment may harbor and transmit *L. monocytogenes* especially in niches such as food processing equipment, floor, drains and points inaccessible for cleaning and sanitizing (Dalton et al., 1997; Fenlon et al., 1996). Subsequently, due to its ability to survive extreme conditions of temperature, salt and pH, and the ability to form biofilm, it may colonize surfaces of processing equipment and form a reservoir of cells that can cross-contaminate food products (Blackman and Frank, 1996; Cole et al., 1990, Hoelzer et al., 2011). In addition, such cells in biofilms have been shown to be more resistant to cleaning and disinfection (Pan et al., 2006), which renders preventing *L. monocytogenes* contamination even more difficult.

Food processing plants.

Food processing equipment may serve as a primary source of *L. monocytogenes* that cross-contaminates food products (Lin et al., 2005). The complexity of food processing environments, as well as poorly designed equipment (Gibson and Holley, 2000) and the presence

of nutrients and humidity in niches suitable for microbial growth may favor the growth and proliferation of *L. monocytogenes* (Chasseignaux et al., 2002 ; Somers and Wong, 2004). Chasseignaux and others (2002) reported the presence of *L. monocytogenes* in the food processing environment and on equipment in raw poultry meat and raw pork meat processing plants. They associated the presence of *L. monocytogenes* with organic residues and surfaces with high relative humidity (above 70 %). They also reported *L. monocytogenes* on the floor, working tables and on the transport belt. Although *L. monocytogenes* was reported to grow at a low pH, in Chasseignaux's study, *L. monocytogenes* growth was more predominant at neutral pH, and temperature below 10° C.

Unpasteurized (raw) milk and cheeses and other foods made from unpasteurized milk are likely to contain *L. monocytogenes* (CDC, 2011). A documented large outbreak in California in 1985 involved Mexican-style cheese made from inadequate pasteurization of milk or by the mixing of raw milk with pasteurized milk (Swaminathan and Gerner-Smidt, 2007). Contamination of the raw milk entering processing plants can be a source of contamination of final products. Latorre and others (2008) reported *L. monocytogenes* was most often associated with the inline filter samples and bulk milk tanks in New York milk farms they visited from 2004 to 2007. *L. monocytogenes* has been isolated in other processing plants including other food processing plants such as fish products and sausages (Gounadaki et al., 2007; Nakamura et al., 2005; Norton et al., 2000).

L. monocytogenes presence in the RTE food establishments is of great concern because RTE foods are consumed without further heat treatment (Gallagher, 2003). With extended storage at retail or during consumers' refrigeration sufficient time may be available for low level pathogen contamination to multiply and reach infective dose concentrations (Chen et al., 2003;

Swaminathan and Gerner-Smidt, 2007). The presence of *L. monocytogenes* on floors, drains, and surfaces of equipment can be the source of food products contamination (Lin et al., 2005). In addition, improper hygienic practices such as sharing utensils in the RTE food industry can expose *L. monocytogenes* to food contact surfaces, which can lead to contamination of RTE foods that had already been subjected to lethality treatment (Hoelzer et al., 2011).

Indeed many RTE food products have been implicated in human listeriosis outbreaks. For example, a multistate listeriosis outbreak occurred in the U.S in 2003 which resulted in 4 deaths and 3 miscarriages and 16 million pounds of RTE meat that was recalled (REF). The source of *L .monocytogenes* was traced to delicatessen turkey RTE meats (Olsen et al., 2005). More outbreaks linked to RTE foods have been reported, such the outbreak that involved Mexican-style cheese in California in 1985. The outbreak resulted in listriosis infections in perinatal infants, and the vehicle of the pathogen was identified as cheese made from inadequately pasteurized milk or pasteurized milk mixed with raw milk (Swaminathan and Gerner-Smidt, 2007). Additionally, outbreaks implicated pate´in the U.K in 1987 to 1999, as well as in France in 1992 and 1993 (Swaminathan and Gerner-Smidt, 2007).

Practices that have been reported to favor transmission of *L. monocytogenes* in RTE foods include improper hand washing (Ross 1999; Clayton and Griffith 2004), handling unwrapped raw foods without proper protective gloves (Angelillo et al., 2006), not changing gloves with sufficient frequency (Green et al., 2006), using the same utensils in handling uncovered salad vegetables (Sagoo et al., 2003), and using the same area in handling raw and cooked or RTE meats (Littke and de Louvois 1998). In addition, sharing utensils in the deli environment has been reported to contribute to *L. monocytogenes* outbreaks (Dorozynski, 2000; Jacquet et al., 1995; Lianou and Sofos, 2007). Such practices have also been reported to cause

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the loss of microbial control in small scale facilities that produce fermented sausages (Gounadaki et al., 2008).

Colonization of surfaces of food service equipment by *L. monocytogenes* can also be the source of cross-contamination of food. Hoelzer and others (2011) studied prevalence of *L. monocytogenes* in 120 small vs large retail establishments, and 4 to 15 % all establishments, regardless of size, tested positive for *L. monocytogenes*. The study demonstrated the ability of *L. monocytogenes* to survive on a range of surfaces including the slicer, cutting board, deli case, sinks, and cold room floor. Hoelzer and others (2011) also found that the odds to find *L. monocytogenes* in small establishments were twice as high for large establishment which emphasize the risk associated with RTE foods produced smaller establishments.

Survival of *L. monocytogenes* on the surface of equipment such as the deli meat slicer increases the probability of the pathogen to be transferred into food products. Lin and others (2006) studied the dynamics of cross-contamination between processing equipment and deli meat, and the authors concluded that *L. monocytogenes* could be transferred from the commercial slicer onto the deli meat and the transferability was positively correlated to the level of the initial inoculum of the pathogen on the equipment. Similarly, Sheen (2008) indicated that *L. monocytogenes* could be transferred from slicing machine onto salami and transferability was affected by the initial inoculum level of the pathogen. The author also proved that the artificially contaminated salami could transfer the pathogen onto the slicing machine; this would infer that the subsequent food product to be sliced could be contaminated. In both cases, the quantity of the pathogen transferred was decreasing according to the number of slices in an unpredictable pattern (sheen, 2008). The above examples highlight the safety concerns associated with deli slicers widely used in retail establishments within the food industry.

Biofilm definition and formation

   L. monocytogenes is able to form biofilms, which contribute to its persistence in the food processing environment (Blackman and Frank, 1996). *L. monocytogenes* was reported to adhere to many materials found in the food-processing environment including stainless steel, aluminum and conveyor belt materials (Beresford et al., 2001; Somers and Wong, 2004).

   The term biofilm refers to a community of microorganisms attached to a solid surface with extracellular polymeric substances (EPS) holding the cells together (Costerton et al., 1987). Cell surface characteristics such as hydrophobicity play a critical role in the initial attachment stage (Chae et al., 2006). The initial attachment is characterized by hydrophobic interactions between the cell surface and the substratum (Chmielewski and Frank, 2003). Nguyen and others (2011) demonstrated that Campylobacter cell surface hydrophobicity correlated positively with attachment to stainless steel and glass, similarly Dickson and Koottmarie (1989) indicated a positive correlation between the hydrophobicity of each of the following bacteria : *Bacillus subtilis*, Escherichia coli O157:H7, *L. monocytogenes*, *Salmonella typhimurium*, *Serratia marcescens*, *Staphylococcus aureus*, and *S. epidermidis* and their corresponding attachment onto fat tissue of beef. The attached cells eventually produce the EPS in which polysaccharides are major component; the EPS attaches irreversibly the cells to the substratum (Surtherland, 1983). As a result, removing such cells from the surface becomes more difficult (Bower al., 1996, Gelinas et al., 1994). Furthermore, it has been reported that cells in biofilms show variation in the gene transcribed than their free-living counterparts and are more resistant to disinfection, sanitization or other antimicrobial substances (Chae et al., 2006; Gravesen et al., 2002; Hefford et al., 2005; Walch et al., 2001), and this may help explain why *L. monocytogenes* is able to survive in the food processing environment (Wong 1998; Holah et al., 2004; Chae et al., 2006).

Pan and others (2006) reported that *L. monocytogenes* biofilm demonstrated increased resistance towards peroxide and other sanitizers, such as quaternary ammonium compounds and chlorine.

L. monocytogenes is able to form biofilms in different niches within the food- processing environment including benches, machinery, and floor (Blackman and Frank 1996). Some *L. monocytogenes* serotypes survive better in specific environments than others. For example, *L. monocytogenes* serotypes 1/2 a, 1/2 b and 1/2 c were isolated frequently in the meat processing environment, which lead to the conclusion that they adapted better in such environments than other serotypes (Jay, 1996; Thevenot et al., 2005). Borucki and others (2003) tested eighty *L. monocytogenes* isolates for biofilm formation and their relation to exopolysaccharide carbohydrate production, and found that serotype 1/2 a showed the greatest biofilm formation, and produced the highest levels of exopolysaccharides.

Separate studies explored the association of attachment and biofilm formation of *L. monocytogenes* to its persistence in the food-processing environment. Borucki and others (2003) reported that *L. monocytogenes* persistent isolates reached a higher number of cells per surface area than sporadic isolates; however Djordjevic and others (2002) did not find such differences when the cells were grown in modified wilshimer's broth, a chemical definied medium. Folsom and others (2006) demonstrated that *L. monocytogenes* serotype 1/2 a strains frequently located in food-processing plants reached higher a density than 4 b strains when grown in 1:10 diluted Trptic Soy Broth (TSB). Similar results were reported by Pan and others (2009) who used 1:10 diluted Triptic Soy Broth with Yeast Extract, and concluded that using a representative media with nutrient concentration of actual field conditions on food processing surfaces is the right approach to explain the association of *L. monocytogenes* attachment to its persistence in the food-processing environment.

Properties of *L. monocytogenes* related to the attachment to stainless steel, a material widely used to manufacture food processing equipment, lead to the resistance to disinfection and to the persistence in the food-processing environment, which create a constant concern of RTE foods cross-contamination in the deli environment (Beresford et al., 2001; Carpentier and Cerf, 2011; Crandall et al., 2011). *L. monocytogenes* attaches to stainless steel, a common material used for equipment such as slicers, knives, work surfaces and other machinery (Harveya et al., 2007). *L. monocytogenes* also attaches to aluminum, which is used to make the blade guard of the slicer or other materials located in the food-processing environment. This implies that cells attached to aluminum surfaces may be a source of cross- contamination (Beresford et al., 2001; Crandall et al., 2011). Besides the inherent ability of *L. monocytogenes* to attach to a meat slicer, there are also other factors in the deli environment that favor the opportunist *L. monocytogenes* to attach to the deli slicer. These include nutrients or humidity availability (Chmielewski and J.F. Frank, 2003; Crandall et al., 2011; Somers and Wong, 2004), and repeated use of the food-processing equipment may cause abrasion on its surfaces, which increase soil and bacteria entrapment (Frank and Chmielewski, 2001; Holah and Thorne 1990). The ability of the pathogen to survive and find niches in the food-processing environment in spite of cleaning and disinfection means that new control strategies are needed.

Control of *L. monocytogenes* biofilms

It is difficult to control biofilms because conditions found in the food-processing plants such as wet surfaces and cold temperatures that promote their formation. Therefore, the most effective control strategies are geared to effective cleaning and sanitization before biofilms can be formed on food contact surfaces (Frank and Koffi, 1999; Midelet and Carpentier, 2004; Simoes et al., 2006). Today there is considerable interest in "green technologies" because of the

pressure to minimize the use of chemical sanitizers and the resistance of *L. monocytogenes* to sanitizing agents, which makes research on preventing *L. monocytogenes* biofilms using green technologies such as competitive exclusion between non-harmful bacteria and the pathogen or using phages even more important.

Cleaning and disinfection

Cleaning

Cleaning serves to remove food residues or mineral build-up from surfaces within the food-processing environment (Simoes et al., 2010). Alkali compounds, alkali in combination with sequestrants or chelators and anionic wetting agents are typically used (Chmielewski and Frank, 2003). Lewis (1980) recommended cleaning stainless steel with alkali or non-ioninic deteregents. Cleaning cannot be the sole reliable procedure to ensure a microbe free environment in the processing area because it only removes 90 % of resident bacteria; consequently, the remaining bacteria can form biofilms whenever there is water or nutrients available (Gibson et al., 1999). Once a surface has had the organic matter removed by cleaning, the subsequent sanitation procedure aims at inactivating any microorganisms remaining on surfaces after cleaning (Dunsmore and Thomson, 1981).

Disinfection/ Sanitation

Disinfectants including halogens, peroxygen, acids and quaternary ammonium are used to sanitize surfaces in the food industry (Gibson et al., 1999). Chlorine is able to remove Listeria or Salmonella EPS from stainless steel; however, the presence of food materials can hamper the effectiveness of chlorine. Quaternary ammonium compounds are also reported to inactivate *L.*

monocytogenes at the concentration of 500 ppm for 5 min and are recommended to use for the floors, walls, storage container and on non-food contact surfaces in general (Giese, 1991).

Peroxides are also effective against *L. monocytogenes* biofilms (Harkonen et al., 1999). Effectiveness of a sanitizer is achieved in the presence of low organic load or a biofilm free environment; however, finding such conditions in the food-processing environment is difficult. *L. monocytogenes* is able to develop resistance to sanitizers. Pan and others (2006) indicated that *L. monocytogenes* in biofilm is able to resist peroxide (100 ppm), quaternary ammonium compounds (150 ppm) and chlorine (200 ppm). Crandall and others (2012) also demonstrated that sanitizer agents were not able to cause a reduction of more than 1.5 CFU/cm2 of *L. monocytogenes* or *L. innocua* adherent to stainless steel or aluminum surfaces found on a meat deli slicer. Additionally, *L. monocytogenes* strains isolated from food-processing plants may develop resistance to the minimum inhibition concentration of quaternary ammonium compounds (Mereghetti et al., 2000). Inability of sanitizers to completely remove *L. monocytogenes* cells from surface, and documented outbreaks linked to *L. monocytogenes* from surfaces of equipment as well as demand of food produced under green conditions has lead researchers to think about alternative bio-based control strategies (Gould, 2000; Zhao et al., 2004). In the food industry, sanitation refers to creating and maintaining hygienic and healthfull conditions (Marrioat, 1997)

Control of *L. monocytogenes* biofilms using phages.

Phages are potential biological agents that can be used to control biofilms in the food-processing environments. Bacteriophages are ubiquitous and pose desirable properties such as

being natural, highly specific, and non-toxic which can be exploited in controlling several microorganisms in biofilms (Kudva et al., 1999). Siringan and others (2011) used bacteriophages CP8 and CP 30 to control virulent strains of *Campylobacter jejuni* biofilms on glass. Bacteriophages were able to reduce up to 3 log CFU/cm^2 of the pathogen in biofilm in contrast to less than 1 log CFU/ml in planktonic cells after 24 hr. Similarly, Soni and Nannapaneni (2010) reported the ability of a bacteriophage P100 to reduce significantly a biofilm of 21 *L. monocytogenes* strains. The bacteriophage reduced 3.5 to 5.4 log/cm^2 *L. monocytogenes* cells in biofilms formed on stainless steel surface (Soni and Nannapaneni, 2010). Nevertheless, research is still needed to address limitations of phage use including ability of *L. monocytogenes* to develop resistance against phages. Moreover, controlling conditions such as chemical composition and the environmental factors, such as temperature, growth stage, media and phage concentration, which affect the infection of the biofilms by the phages needs to be addressed (Chaignon et al., 2007; Sillankorva et al., 2004).

Control of *L. monocytogenes* biofilm using competitive microorganisms

Microbial competition

Competitive exclusion refers to competition between two or more microorganisms for nutrients or resources in the environment (Fredrickson and Stephanopoulos, 1980). One population of microorganisms may produce and secrete metabolites that hinder the growth of the competitor. When such microorganisms are surviving on a single resource, competition may lead to elimination of the weakest. Conversely, one population may also contribute to the proliferation of the others (Fredrickson and Stephanopoulos, 1980). Interaction of several microorganisms within biofilms may interfere with their development (Carpentier and Chassing,

2004; Kives et al., 2005). Norwood and Gilmour (2001) observed a decrease of *L. monocytogenes* cell numbers attached on stainless steel at 4, 18, and 30° C in the presence of *Staphylococcus xylosus* and *Pseudomonas fragi*. The decrease was attributed to the competition of nutrients and production of antagonistic compounds by *S. xylosus*. In addition, Villani and others (1997) reported that *S. xylosus* produces heat stable protein substances inhibitory to *L. monocytogenes*. A separate study done by Leriche and Carpentier (2000) asserted that *Staphylococcus* could produce extracellular polysaccharides that may contain teichoic acid, which prevent adhesion of bacterial cells to a substratum.

However, Sasahara and Zottola (1993) observed an increase of the attachment and biofilm development of *L. monocytogenes* in flowing systems on glass coverslips in presence of *P. fragi*. The attachment of *L. monocytogenes* in the mixed-cultures was enhanced by the exopolymer produced by *P. fragi* compared to the *L. monocytogenes* in pure culture. Moreover, Guerrieri et al., 2009 demonstrated *P. fragi* can increase the pH, which render antilisterial substances such as those produced by *Lactobacillus sp* inactive, and as a result, the growth of *L. monocytogenes* in the medium is favored. Similarly, *Flavobacterium sp* is able to increase the attachment of *L. monocytogenes* to stainless steel. In addition, *L. monocytogenes* cells co-cultured with *Flavobacterium sp* were able to persist on a stainless steel surface for a long periods of time. In monoculture biofilms, the decimal reduction times were 1.2 and 18.7 days for *L. monocytogenes* in mixed biofilm, respectively (Bremer at al., 2001). Leriche and others (2003) showed that spatial distribution of bacteria in a biofilm could result in one species protecting another from disinfectants. In his experiment, three bacterial strains (*Kocuria* sp., *Brevibacterium linens* and *S. sciuri*) were grown in whey; *Kocuria sp* grew and became the

dominant species in the biofilm. *Kocuria sp* developed resistance to a chlorinated solution of 22 mg/l and eventually protected the growth of *S. scuri* (Leriche et al., 2003).

Competition of bacteria on surfaces and differences in their generation time can also determine which species thrives better. Habimana and others (2011) studied the spatial competition of *Lactococcus lactis* as an example of a resident microorganism on industrial surfaces, and *L .monocytogenes* under continuous flow biofilm. During the initial attachment, the difference in generation times between *Lc. Lactis andL. monocytogenes*,was responsible for the pathogen inhibition. Demand for food processed with minimum use of chemical preservatives has increased attention in LAB research geared to control foodborne pathogens in foods or in the processing environment (Amezquita and Brashears, 2002; Gould, 2000; Guerrieri et al., 2009; Zhao et al., 2004; Zhao et al., 2006). LAB produce natural antimicrobials able to suppress foodborne pathogens (Jay, 1982).   Some LAB strains able to competitively exclude *L. monocytogenes* in meats products have been granted a Generally Recognized as Safe (GRAS) status, which make thes strains the best candidate in improving microbial safety in food and in the environment (Concha-Meyer et al., 2011).

Amezquita and Bashears (2002) reported that *Pediococcus acidilactici* produced bacteriocin like compounds that contributed to the inhibition of the growth of *L. monocytogenes* in RTE meat products. Guerrieri and others (2009) demonstrated that LAB was able to inhibit *L. monocytogenes* growth in planktonic and biofilm by producing bacteriocin, cell to cell competition or production of organic acids that lowered the pH. *Enterococcus casseliflavus* reduced 3.5 log of adherent cells and pathogen reduction was solely attributed to the bacteriocin production whereas *Lb. plantarum* reduced 5.4 log due to both bacteriocin and organic acids. Lastly, *E. faecalis* did not produce bacteriocin, affected the pathogen through cell to cell

competition for access to nutrients (Guerrieri et al., 2009). Indeed, several other LAB strains were used to control *L. monocytogenes* for their properties of producing bacteriocin in foods (Amezquita and Brashears, 2002; Concha-Meyer et al., 2011) and in the niches of food processing environment (Garcia-Almendarez et al., 2007; Zaho et al 2004) . Leriche and others (1999) noticed advantage of using nisin-producind strain *Lc. lactis* to control *L. monocytogenes*. Not only has the nisin played a role in the pathogen reduction, but also the density of *Lc. lactis* out-competed the pathogen on stainless steel surface.

Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore forming, non-respiring cocci or rods grouped by common morphological, metabolic, and physiological characteristics. They produce lactic acid as an end product during fermentation of carbohydrates through the glycolysis pathway (Salminen, 2004). LAB are well known in their role as beneficial intestinal bacteria, as flavor compound producers and as bio-preservatives in dairy and fermented foods (El Bassi et al., 2009; Nandan et al., 2010; Ravyts and Vuyst, 2011). LAB have been used for the control of food pathogens and spoilage microorganisms (Bernardi et al., 2011; Brown et al., 2001). Besides producing organic acids (lactic acid and acetic acid), hydrogen peroxide or diacetyl that are also inhibitory towards competing microorganisms (Jay, 1982), LAB also synthesize low-molecular-weight antimicrobial bacteriocins that suppress the growth and multiplication of competitive microorganisms (Nes et al., 1996). These LAB properties have led researchers to explore the use of LAB to suppress *L. monocytogenes* and other in food products. Amezquita and Brashears, (2002) reported the inhibition of the growth of *L. monocytogenes* by LAB in frankfurters and cooked ham at 5° C over a period of 28 days of incubation. Similarly, Koo and others (2012) used LAB to control the growth of *L.*

monocytogenes in hot dogs formulated without potassium lactate and sodium diacetate. Despite the fact that LAB displayed antagonistic properties towards *L. monocytogenes*, there are few studies that have focused on the control of *L. monocytogenes* attached on stainless steel commonly found in retail food environments using LAB.

Antimicrobial components from lactic acid bacteria

Organic acids

Lactic acid bacteria produce organic acids including lactic acid, acetic acid and propionic acid upon fermentation of carbohydrates (Saliminen, 2004). Antimicrobial activity of these weak acids is strong at a low pH (Simon and Blackman, 1949). It has been demonstrated that acetic acid is the strongest, and is able to inhibit the growth of bacteria, yeast and molds (Soumalainem and Mayra-Makinen, 1999). Propionic acid also exerts antimicrobial activity against yeast and molds and can act synergistically with acetic acid to inhibit Gram-negative bacteria such as *Salmonella enterica ser. typhimurium* (Robin, 1979). Lactic acid not only reduces the pH which renders the medium unfavorable for the growth of other microorganisms but also permeabilizes bacterial membranes, which enhances the activity of other antimicrobial agents (Alakomi et al., 2000).

Hydrogen peroxide

Lactic acid bacteria use enzymes such as flavoprotein-containing oxidases, NADH oxidases, and superoxidase dismutase, to produce hydrogen peroxide in presence of oxygen (Condon, 1987). Antimicrobial activity of hydrogen peroxide results from its oxidizing effect on the bacterial cell; sulfhydryl groups of cell protein and membrane lipids can be oxidized. In

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addition, some hydrogen peroxide reactions can create anaerobic environments unfavorable for the growth of many microorganisms (Saliminen, 2006).

Diacetyl

LAB genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* also produce diacetyl with a demonstrated antimicrobial activity against Bacillus sp (Jay, 1982). Van Neil and others (1929) identified diacetyl as aroma and flavor in butter. The antimicrobial activity of diacetyl is optimum at a pH below 7, and is more active against Gram-negative bacteria (Jay, 1982). Diacetyl reacts with arginine binding protein, as a result, the utilization of the amino acid is obstructed.

Bacteriocin

Bacteriocins are ribosomally synthesized proteinaceous substances produced by LAB that suppress the growth of other bacteria. They are characterized by a narrow killing spectrum and their antimicrobial activity is generally against related species (Jack et al., 1995). Bacteriocins have gained applications in the food industry because they regarded as natural, a trait highly attractive to many consumers. Bacteriocins can be divided into three classes according to their biochemical and genetic characteristics: lantibiotics, non –lantiobiotic/small heat-stable peptides, and large heat sensitive molecules. The first two classes are the most commonly explored for commercial applications because of their abundance (Jack and Sahl, 1995). Lantibiotics are small peptides consisting of unusual amino acids such as lantionine, methyl-lanthionine, dehydrobutyrine and dehydroalanine. Lantibiotics can be further divided into two subclasses: type A whose activity disrupts the membrane integrity of the target organism, and type B which

disrupts enzyme function resulting in destabilization of normal cell processes such as cell wall biosynthesis (McAuliffe et al., 2001).

Class II bacteriocins are heat-stable non-lathionine containing and membrane active peptides, and is divided into 3 subclasses. Subclass IIa is the major sub-group and is also of great interest because they possess antilisterial activity, furthermore, they have a narrow spectrum of activity, which means they do not kill LAB starter cultures. They are designated as pediocin like bacteriocins. *Pediococcus acidilactici*, produces pediocin and is usually used for fermentation of meat and vegetables (Saliminen, 2004).

Conclusions

L. monocytogenes can cause food contamination and listeriosis outbreak because of its ability to attach to and form biofilms on several materials found in food-processing plants. Inability of cleaning and sanitization to remove all cells of *L. monocytogenes* attached to surfaces commonly located in food-processing plants, and the demand of food produced with minimum use of chemicals means green control strategies are needed. The presence of *L. monocytogenes* in the deli environment poses even more concern because RTE foods are eaten without further heat treatment, where the cross-contamination of the pathogen from food processing equipment to food has already been demonstrated. Therefore, attachment of *L. monocytogenes* to equipment must be prevented if the food safety is to be achieved. LAB are ideal candidates to control *L. monocytogenes* not only because they produce antimicrobial substances and can compete with *L. monocytogenes* to specific niches but also LAB are recognized as GRAS. In addition, understanding of *L. monocytogenes* survival and interaction with other microorganisms in the

food-processing environment would enhance the ability to develop green methods to control the

pathogen.

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Role of lactic acid bacteria as a bio-sanitizer to prevent attachment of *Listeria monocytogenes*

F6900 on deli slicer food contact surfaces.

Role of lactic acid bacteria as a bio-sanitizer to prevent the attachment of *Listeria monocytogenes* F6900 on deli slicer food contact surfaces.

Jean Baptiste Ndahetuye, Ok Kyung Koo, Corliss A. O'Bryan, Steven C. Ricke, Navam Hettiarachchy, Philip G. Crandall.

Center for Food Safety, Department of Food Science, University of Arkansas, Fayetteville, 72704

Corresponding author: Dr. Philip G. Crandall

Center for Food Safety, Dept. of food science

 26 50 N Young Ave, Fayetteville, AR 72704

University of Arkansas

E-mail: crandal@uark.edu

Abstract

The study was conducted to evaluate the attachment of three lactic acid bacteria (LAB) strains and their combination in a cocktail to stainless steel coupons from a deli slicer, and their ability to inhibit the attachment of *Listeria monocytogenes*. In a previous study, three LAB strains, *Pediococcus acidilactici*, *Lactobacillus amylovorus* and *Lb. animalis*, exhibited an antilisterial effect. In the study reported here, hydrophobicity tests were determined according to microbial adhesion to solvent (MATS). The attachment of the cells was evaluated on stainless steel coupons cut from deli slicers. Extracellular carbohydrates were determined using a colorimetric method. Based on these tests, *Lb. animalis* exhibited the greatest hydrophobicity (26.3 %) and its adherence increased sharply from 24 to 72 hr, whereas *Lb. amylovorus* yielded the lowest hydrophobicity 3.86 % and was weakly adherent. Although *P. acidilactici* had a moderate hydrophobicity (10.1 %), it was strongly adherent. The attached LAB strains produced significantly ($P < 0.05$) higher total carbohydrates than their planktonic counterparts, which is an important characteristic for attachment. Three conditions were simulated to evaluate the ability of the LAB cocktail (10^8 CFU/ml) to competitively exclude *L. monocytogenes* (10^3 CFU/ml) on the surface of the coupons. In a complete block design, the coupons were pre-treated with either the LAB cocktail for 24 hr prior to the addition of *L. monocytogenes*, simultaneously treated with the LAB cocktail and *L. monocytogenes*, or pre-treated with *L. monocytogenes* 24 hr prior to the addition of the LAB cocktail. The LAB cocktail was able to significantly reduce the attachment *L. monocytogenes* ($P < 0.05$). The LAB cocktail indicated potential attachment on stainless steel and bacteriostatic activity toward *L. monocytogenes* attached on stainless steel, which indicates a possible role for LAB as a bio-sanitizer in the food industry.

Introduction

Listeria monocytogenes is a Gram-positive, facultative-anaerobic, rod-shaped bacterium that the food industry regards as an important foodborne pathogen (Cossart, andToledo-Arana, 2009, García-Almendárez et al., 2008). The organism is responsible for a life-threatening disease "listeriosis" that is able to cause maternofetal- or neonatal-listeriosis, bloodstream infection, and meningoencephalitis. People at high risk are pregnant women, neonate and immunocompromized adults (Swaminathan and Gerner-Smidt, 2007*). L. monocytogenes* is not only a public health burden because of high rates of mortality and high hospitalization associated with listeriosis cases, which are estimated to be 15.9 % and 94.0 %, respectively (Scallan et al., 2011) but also an economic problem for the food industry due to immense food recalls (Gandhi and Chikindas, 2007). The pathogen is ubiquitous in the environment, and it easily disseminates into food processing surroundings including the deli environment, and as a result it may contaminate food products (Møretrø and Langsrud, 2004). Although significant progress in sanitary systems designs in the past few decades have been achieved, *L.monocytogenes* outbreaks continue to occur, and additional control strategies are needed to prevent food contamination.

L. monocytogenes has been isolated in a retail environment where it can persist for long periods of time and become a potential source of cross-contamination of food products (Hoelzer et al., 2011; Wilks et al., 2006). *L. monocytogenes* has the ability to attach to and forms biofilms on materials such as stainless steel, rubber and plastics (Beresford et al., 2001). The attachment of *L. monocytogenes* to stainless steel, a material widely used in the food-processing environment, increases transferability of *L. monocytogenes* into food products (Lin et al., 2007; Wilks et al., 2006). Ready-to-eat foods (RTE) are particularly predisposed to be contaminated

from *L. monocytogenes* in the environment. The RTE foods eventually pose a health risk as they may be consumed without further heat treatment (Sheen, 2008). For instance, Olsen and others (2005) reported an outbreak linked to delicatessen turkey meat that resulted in 5 deaths, 3 miscarriages and 16 million pounds meat recall. Similarly, Graves and others (2005) reported listeriosis outbreak that involved hot dogs, the outbreak caused illness in108 persons, 14 deaths and four miscarriages. Other RTE foods such deli meats, hot dogs, and unpasteurized milk and cheeses, as well as foods made from unpasteurized milk can potentially serve as vehicle for *L. monocytogenes* to humans (CDC, 2011).

L. monocytogenes attachment to materials located in the deli processing environment including slicers is a concern of cross-contamination of RTE food products (Crandall et al., 2011). Microorganisms attached to surfaces have different characteristics than their planktonic counterparts, including genetic expression, higher production of polysaccharides and greater resistance to disinfection, sanitization or other antimicrobial substances (Chae et al., 2006; Gravesen et al., 2002; Hefford et al., 2005; Walsh et al., 2001). Pan and others (2006) reported that *L. monocytogenes* biofilm demonstrates resistance towards peroxide and other sanitizers, such as quaternary ammonium compounds and chlorine. Therefore, preventing the *L. monocytogenes* attached to food equipment is a good approach to prevent food contamination.

Lactic acid bacteria (LAB) are well known for their role as beneficial intestinal bacteria, as flavor compound producers and as bio-preservatives in dairy and fermented foods (El Bassi et al., 2009; Nandan et al., 2010; Ravyts, and Vuyst, 2011). The LAB have demonstrated antagonistic activity against food pathogens and spoilage microorganisms such as *Pseudomonas aeruginosa*, *Escherichia coli* or *Clostridium sporogenes* (Brown et al., 2011; Hatew et al., 2011). Besides producing organic acids (lactic acid and acetic acid), hydrogen peroxide or diacetyl that

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are inhibitory towards microorganisms (Jay 1982). LAB also synthesize low-molecular-weight antimicrobial substances such as bacteriocins or reutinin that suppress the growth and multiplication of competitive microorganisms (Arqués et al., 2011). These LAB properties have led researchers to explore the use of LAB to suppress *L. monocytogenes* in food products. Amezquita and Brashears, (2002) reported the inhibition of the growth of *L. monocytogenes* by LAB in frankfurters and cooked ham at 5° C over 28 days of incubation. Similarly, Koo and others (unpublished) used LAB to control the growth of *L. monocytogenes* in formulated hot dogs without sodium lactate and sodium diacetate. Despite the fact that LAB displayed antagonistic properties towards *L. monocytogenes*, studies on the control of *L. monocytogenes* attached to stainless steel using LAB are limited. The present study focused on characterization of the attachment of three LAB strains, *Lb. animalis*, *Lb. amylovorus* and *P. acidilactic*i, their combination in a cocktail, and *L. monocytogenes* by examining their cell surface hydrophobicity, total carbohydrate production and adherence capability to stainless steel at room temperature. Subsequently the study evaluated the ability of the LAB cocktail to inhibit the attachment of *L. monocytogenes* to stainless steel.

Material and methods

Bacterial strains, media and culture conditions. *L. monocytogenes* F6900 (serotype 1/2a) (Cornell University, Ithaca, NY). Serotype 1/2 a was selected because of it has been previously reported to have the greatest attachement and production of extracellular polysaccharides (Borucki and others 2003). Three LAB strains: *Lb. animalis*, *Lb. amylovorus* and *P. acidilactici* which are sold under the commercial name LactiGuard (Guardian Food Technologies, Overland Park, KS) were used in this study. *L. monocytogenes* was cultured in tryptic soy broth supplemented with 0.6 % yeast extract (TSB-YE) (Becton, Dickinson & Co, Sparks, MD) at 37°

C for 18 to 20 hr and the LAB strains were cultured in de Man Rogosa Sharpe (MRS) broth (EMD Chemical Inc., Gibbstown, NJ) 37° C for 18 to 20 hr. All strains were maintained frozen at – 80°C in their respective broth containing 20 % glycerol.

Stainless steel coupons. Stainless steel coupons (1.8 x 2.4 cm) cut from the blade of a Hobart Heavy deli slicer (Hobart Food Equipment, Silverwater, Australia) were used as a model of a food contact surface. Coupons were washed thoroughly with a dish detergent, rinsed well, wrapped in aluminum foil and autoclaved at 120°C for 15 min before use.

Cell-surface hydrophobicity. The cell surface hydrophobicity was determined according to the microbial adhesion to solvent procedure (MATS) (Bellon-Fontaine et al., 1996). The affinity of the bacterial cells to the organic solvent n-hexadecane (Sigma-Aldrich Co, St. Louis, MO) was determined. The LAB strains, and their combination in a cocktail (LAB cocktails consisted of 2: 1: 1 ratio of *P. acidilactici*: *Lb. amylovorus*: *Lb animalis*) and *L. monocytogenes* cultures were harvested by centrifugation at 7,000 x g for 5 min for three times and they were resuspended in phosphate buffer saline (PBS) to an absorbance (OD620) of one using spectrophotometer (Model: DU 600 series, Beckman Instruments Inc., Fullerton, CA). Subsequently, 1 ml of n- hexadecane was added to 1 ml of each bacterial suspension and the mixture was incubated at 30° C for 10 min. After vortexing for 60 sec, the two phases were left to separate for 15 min. The OD620 nm of the volume that was drawn from the aqueous phase was recorded. The hydrophobicity was given by the following formula [(1-OD620 after mixing)/OD620 before mixing] x 100. There were nine independent trials, and each trial consisted of one replicate.

Quantitative analysis of extracellular total carbohydrates. The carbohydrates produced were analyzed from both planktonic and attached cells supernatant according to phenol –H2SO4 method described by Dubois and others (Dubois et al., 1956). *L. monocytogenes*, three strains of LAB, and LAB cocktail were grown as described above. Cells were washed with PBS three times by centrifugation (3,000 x g at 4° C for 10 min) and their absorbance (OD600) was adjusted to 0.3 OD Individual cell suspensions of 5 ml were incubated at 23° C for 3 hr. After incubation, cell suspensions were centrifuged at 3,000 x g at 4° C for 10 min, 2 ml of the supernatant were used for carbohydrate analysis. Simultaneously, a volume of 5 ml of each bacterium suspension was deposited on stainless steel coupons in a sterile petri dish, subsequently the coupons were incubated at 23° C for 3 hr. After incubation, the coupons were rinsed with phosphate buffered saline (PBS) to remove loosely bound planktonic cells. The attached cells were removed by swabbing the surface of the coupons for approximately 100 times using sterile polyester tipped applicators (Puritan Medical Products Company LLC, Guilford, ME). The swab was placed in 5ml PBS and vortexed. The tube containing the swab and the bacteria suspension were centrifuged at 3,000 g at 4° C for 10 min. Two milliliter of the supernatant was extracted for carbohydrate analysis. Eighty percent phenol 0.05 ml and 5ml of 96% sulfuric acid were added sequentially to 2 ml of planktonic and supernatant of the standard. Samples were left at room temperature for 10 min, and each was mixed before incubation at 28° C for 20 min. The absorbance at 460 nm was measured. The total carbohydrates of each sample were determined from a glucose standard curve. There were three independent trials and each trial was done in duplicate.

Bacterial attachment on deli slicer surfaces. The method described by Norwood and Gilmour (32) was adapted for bacterial attachment to stainless steel. The three LAB strains, their

cocktail and *L. monocytogenes* were grown as previously described. The coupons were placed into 6-well plates filled with TSB-YE. LAB (10^8 CFU/ml), and *L. monocytogenes* (10^3 CFU/ml) were inoculated in separate wells. Afterwards, the plates were pre-incubated at 23° C for 3 hr to allow the bacteria to attach to the coupons. Thereafter, the coupons were washed carefully with 1 ml PBS three times in order to remove loosely bound cells. The coupons were placed in a fresh TSB-YE followed by incubation at 23° C for 24 and 72 hr. After each incubation period, the coupons were aseptically recovered and rinsed with PBS three times. They were placed into tubes containing 15 ml PBS. Subsequently, the coupons were sonicated (Misonix Incorporated, Farmingdale, NY) in a bath for 3 min at 50 % amplitude to release bound cells. Appropriate dilutions were dispensed in duplicate on MRS plates for LAB strains and on modified oxford agar (MOX) (EMD Chemical Inc., Gibbstown, NJ) plates for *L. monocytogenes* count. MRS and MOX plates were incubated at 37° C for 24 and 48 hr, respectively and enumerated. The coupons that were not treated by either bacterium served as negative controls. There were 3 independent trials and each trial had triplicates.

Competitive exclusion of *L. monocytogenes* by LAB. The LAB cocktail and *L. monocytogenes* were grown to the stationary phase as previously described. The coupons were placed in 6-well plates containing 6 ml of TSB-YE. The LAB cocktail concentration of 10^8 CFU/ml was inoculated into wells, and the plate was pre-incubated at 23° C for 3 hr to allow bacteria to attach to the coupons. After incubation, each coupon was washed with PBS three times, placed in a fresh TSB-YE and incubated at 23° C for 24 hr. For inhibition of *L. monocytogenes* attachment by the LAB cocktail, 10^3 CFU/ml of *L. monocytogenes* was inoculated to the LAB cocktail pre-treated coupons contained in fresh TSB-YE. Samples were pre-incubated at 23° C for 3 hr; then washed with PBS three times. Subsequently they were

incubated at 23° C for 24 and 72 hr. The incubation period was considered from the time *L. monocytogenes* was added. After each incubation period, the coupons were asceptically recovered and carefully washed with PBS for three times to remove loosely bound cells, transferred to a tube containing 15 ml PBS then they were sonicated in a bath for 3 min at 5 amplitude to release bound cells. *L. monocytogenes* surviving in the presence of the LAB cocktail was quantified. The coupons treated with the LAB cocktail or *L. monocytogenes* alone served as controls.

Minimum LAB cocktail concentration to inhibit the attachment of *L. monocytogenes* was determined. Serial LAB cocktail concentrations of 10^8, 10^6, 10^4 and 10^2 CFU/ml were prepared. The coupons were pre-treated with each of the LAB cocktail concentration for 24 hr, and 103 CFU/ml *L. monocytogenes* was added. To evaluate the competitive attachment, the coupons were treated simultaneously by *L. monocytogenes* (10^3 CFU/ml) and the LAB cocktail (108 CFU/ml) whereas the displacement attachment was evaluated by pre-treating the coupons with *L. monocytogenes* (10^3 CFU/ml) for 24 hr prior to the addition of the LAB cocktail (10^8 CFU/ml). The cell numbers of *L. monocytogenes* and the LAB cocktail remaining on the coupons after 24 and 72 hr were quantified as previously described. There were 3 independent trials and each trial had duplicates.

Statistical analysis. One-way ANOVA analysis of variance tests were used to test differences among means for treatments at a significance level of 5 %. An ANOM Variance with Levene (ADM) transformation was applied to hydrophobicity to evaluate differences among means. All calculations were performed using JMP statistical software (JMP Pro 9.02) (SAS Institute Inc., Cary, NC)

Results

Cell surface hydrophobicity and total carbohydrates production.

Based on cell affinity to the organic solvent n-hexadecane, the hydrophobicity of the cells ranged from 3.86 % to 26.3 % ($P = 0.05$). Among LAB strains, *Lb. animalis* had the highest hydrophobicity (26.3 %), whereas *Lb. amylovorus* had the lowest hydrophobicity (3.86 %). The hydrophobicity of the *P. acidilactici* was 10.1 %. The hydrophobicity of the combination of the three LAB strains in the cocktail was 19.7 %, which was higher than of *L. monocytogenes* (10.8 %). Overall, the total carbohydrates production measured after a 3-hr incubation of the attached cells was significantly higher ($P < 0.05$) than those of planktonic cells over the same incubation time. Total carbohydrate production varied from 0.60 to 0.69 mg/log CFU among the attached cells and from 0.50 to 0.64 mg/log CFU among the planktonic cells (Table 2). Within planktonic cells, *Lb. animalis* produced 0.55 mg/log CFU which were higher than those of *P. acidilactici* (0.50 mg/log CFU) and *Lb. amylovorus* (0.54 mg/log CFU). The planktonic of the LAB cocktail produced significantly higher amounts of carbohydrates than individual LAB strains ($P < 0.05$).

Attachment of *L. monocytogenes* and three LAB strains.

The results are presented in Table 3 indicated that among the LAB strains, *P. acidilactici* exhibited the greatest attachment with viable counts of 8.60 and 8.85 log CFU/cm^2 for 24 and 72 hr, respectively. Its attachment was significantly greater ($P < 0.05$) than the attachment of *Lb. amylovorus* after 24 and 72 hr. The attachment of *P. acidilactici* was also significantly higher ($P < 0.05$) than the attachment of *Lb. animalis* after 24 hr. *Lb amylovorus* showed the lowest attachment with viable counts of 4.03 log CFU/cm^2 and 3.79 log CFU/cm^2 for 24 and 72 hr, respectively. *Lb. animalis* was the only LAB strain to increase significantly ($P < 0.05$) from 24 to

72 hr. At the level of inoculum of 10^8 CFU/ml for the LAB cocktails and 10^3 CFU/ml for *L. monocytogenes*, both inocula demonstrated similar level of attachment ($P > 0.05$) as shown in Table 3. Viable counts were 8.60 log CFU/cm^2 and 9.09 log CFU/cm^2 for the LAB cocktails and 8.44 log CFU/cm^2 and 9.67 log CFU/cm^2 for *L. monocytogenes*, respectively.

Competitive exclusion of *L. monocytogenes* by LAB

Inhibition attachment of *L. monocytogenes* by LAB. The LAB cocktails significantly reduced ($P < 0.05$) *L. monocytogenes* attachment. The pathogen was reduced by 2.82 log after 24 hr of incubation on the LAB cocktails treated coupons compared to the non-treated coupons. Pathogen reduction was even greater (3.81 log) after 72 hr compared to the control (Figure 1A). The LAB cocktail control only increased by 0.23 log from 24 to 72 hr (Figure 1B). There were no significant differences ($P > 0.05$) between the LAB cocktails populations in the control samples and the LAB cocktails populations in the presence of *L. monocytogenes* on the coupons.

An additional inhibition test was carried out to determine the lowest concentration of the LAB cocktails to elicit an attachment inhibition effect against a *L. monocytogenes* concentration of 103 CFU/ml. The results demonstrated that 10^6 CFU/ml was the lower limit of the LAB cocktail concentration that significantly reduced ($P < 0.05$) the attachment of the pathogen at both sampling times (Figure 1C).

Competitive attachment between *L. monocytogenes* and LAB. The LAB cocktails also had a significant inhibitory effect ($P < 0.05$) on the attachment of *L. monocytogenes* when both organisms were added to the coupons at the same time. The LAB cocktails reduced the attachment of the pathogen by 4.38 log after 24 hr, and by 3.22 log after 72 hr (Figure 2A). *L. monocytogenes* positive control increased by 1.07 log over the tested incubation period whereas

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the LAB cocktails control only increased by 0.18 logs over the same incubation period in this experiment (Figure 2B). The LAB cocktails in the treated samples and the LAB cocktails in the control samples did not differ significantly ($P > 0.05$) after 24 and 72 hr. When the LAB cocktails concentration of 10^6 CFU/ml was co-inoculated with a *L. monocytogenes* concentration of 10^3 CFU/ml on the coupons, there were no significant pathogen reduction ($P > 0.05$). Nevertheless, *L. monocytogenes* decreased by 0.21 log after 24 hr and by 1.75 log after 72 hr (data not shown).

Displacement attachment of *L. monocytogenes* by LAB. On these stainless steel surfaces contaminated with *L. monocytogenes*, the LAB cocktail significantly displaced ($P < 0.05$) *L. monocytogenes* after a 24-hr contact time. Results demonstrated that L. monocytogenes was decreased by 2.51 log (Figure 3A) compared to the *L. monocytogenes* positive control after a 24-hr contact time. The LAB cocktail was still able to reduce the pathogen by 1.79 logs after 72 hr. The LAB cocktail in the treated samples and the LAB cocktail in the control did not differ significantly ($P > 0.05$) (Figure 3B). When the LAB cocktail concentration was reduced to 10^6 CFU/ml and tested against a *L. monocytogenes* concentration 10^3 CFU/ml, there was no statistically significant attachment inhibitory effect ($P > 0.05$). However, *L. monocytogenes* was lowered by 1.3 log after 72 hr. (data not shown).

Discussion

Control of the food-borne pathogen *L. monocytogenes* is a challenging task for the food industry (Zhao et al., 2004). One of the factors that contribute to *L. monocytogenes* outbreaks is its ability to attach to surfaces present in food- processing environments, which increases its survivability and subsequent food contamination (Wilks et al., 2006). It has also been reported

that *L. monocytogenes* cells attach to stainless steel, a material widely used in the food industry (Beresford et al., 2001). Lactic acid bacteria, beneficial organisms known to produce antimicrobial substances, have been sought for control of food spoilage and pathogenic microorganisms and studies have explored the use of LAB to control *L. monocytogenes* in food products (Amezquita and Brashears, 2002). The outcome of this study demonstrated the ability of three LAB strains and their combined cocktail to attach to a stainless steel surface and prevent or displace *L. monocytogenes* on a stainless steel surface.

It is established that bacteria attachment to abiotic surfaces depends on the cell characteristics such as hydrophobicity and extracellular substances (Roosjen et al., 2006). Dickson and Koottmarie (1989) reported a positive correlation between the hydrophobicity of each of the following bacteria: *Bacillus subtilis*, *Escherichia coli* O157:H7, *L. monocytogenes*, *Salmonella Typhimurium*, *Serratia marcescens*, *Staphylococcus aureus*, and *S. epidermidis* and their corresponding attachment onto fat tissue of beef. According to Li and McLandsborough, (1999) cellular hydrophobicity can be classified into the following categories: strongly hydrophobic (over 55%), moderately hydrophobic (30 to 54%), moderately hydrophilic (10 to 29%), and strongly hydrophilic (below 10 %). The hydrophobicity determined in the current study according to microbial adhesion to hexadecane, ranged between 3 and 27% suggesting a hydrophilic character for these bacteria. The results showed *Lb. amylovorus* was strongly hydrophilic and was weakly adherent whereas *Lb. animalis* was moderate hydrophilic and its attachment to stainless steel increased sharply from 24 to 72 hr compared to the other LAB strains. The hydrophobicity of *P. acidilactici* was moderate even though it showed the greatest attachment level compared to the rest LAB strains. The LAB cocktails and *L. monocytogenes*

were moderate hydrophilic. These results prompted further testing of the attachment of both bacteria and evaluation of their competitive exclusion capabilities on stainless steel.

Extracellular polysaccharides substances (EPS) function as cell receptors in the process of bacterial attachment to epithelial cells and to abiotic surfaces (Adlerberth et al., 1996). Cammarota and Sant'Anna (1998) reported that blocking the carbohydrate synthesis pathway resulted in reduced bacteria adhesion to glass slides, and the authors established a direct relationship between carbohydrates production and biofilm formation. EPS consist primarily of carbohydrates; consequently, measuring the carbohydrates produced by the bacterial cells should reflect the overall EPS contents (Li et al., 2006). It is also known that the attached bacteria produce more carbohydrates than their free-living cell counterparts (Sutherland, 2001). It is from this premise that the total carbohydrates produced by the three LAB strains, their combination in a cocktail and *L. monocytogenes* were quantified after a 3-hr attachment to stainless steel compared to their planktonic cell counterparts. The results of this study corroborated to those of Chae and others (2006). The cells attached to stainless steel coupons produced more carbohydrates than the free-living cell counterparts; this is an important property because it shows the ability of bacteria to form a biofilm (Sutherland, 2001). Similar to hydrophobicity, the carbohydrates produced by the LAB cocktail and *L. monocytogenes* were not statistically different ($P > 0.05$).

L. monocytogenes has been previously reported to attach to materials present in the food - processing environment (Beresford et al., 2001). The attachment to materials particularly originating from food retail equipment such as sandwich slicers increases the likelihood of *L. monocytogenes* transfer to RTE food products (Lin et al., 2007). Among the LAB strains, *P. acidilactici* exhibited the greatest ability to attach to stainless steel followed by *Lb. animalis* then

Lb. amylovorus. At the inoculum level tested, the LAB cocktails and *L. monocytogenes* showed a comparable ability to attach to the coupons, and although their respective attachments to the coupons were not statistically different ($P > 0.05$); the result showed that the LAB cocktails could be used to compete with *L. monocytogenes* on attachment to stainless steel. In addition, individual LAB strains may have contributed the overall observed hydrophobicity, carbohydrate production and the attachment properties of their combination in a cocktail.

When competitive exclusion between the LAB cocktails and *L. monocytogenes* was investigated, the results demonstrated that the sequence of bacterial treatment to the coupons influenced the degree of pathogen inhibition. For prevention of attachment, the LAB cocktails could have inhibited *L. monocytogenes* through a combination of different mechanisms; notably the LAB cocktails coated on the surface of the coupons effectively inhibited the attachment of incoming *L. monocytogenes*. In addition, *P. acidilactici* had been previously characterized for production of a bacteriocin-like compound that exhibited antilisterial activity (Amezquita and Brashears, 2002). Thus *P. acidilactici* metabolites may also have played a role in the pathogen reduction. Because *L. monocytogenes* can develop resistance after being co-inoculated with bacteriocin producer cells due to production of proteolytic enzymes that degrade inhibitors (Duffes et al., 1999), coating the LAB cocktails on the coupons prior to the addition of *L. monocytogenes* could have prevented *L. monocytogenes* access to the area of attachment on the surface of the coupons. When lower concentrations of the LAB cocktails were used, the results confirmed 10^4 CFU/ml to be the limit concentration that reduced significantly ($P < 0.05$) the attachment of the pathogen suggesting the LAB cocktails and *L. monocytogenes* concentrations are critical in determining the inhibition levels.

The LAB cocktails was also able to directly compete with *L. monocytogenes* on the attachment to the surface and exclude the pathogen significantly ($P < 0.05$) when they were added simultaneously on the coupons. Production of antilisterial compounds by the LAB cocktail may have enhanced the LAB cocktails' capability to competitively exclude *L. monocytogenes* on the coupons. Furthermore cell to cell competition on the available space for the attachment appeared to hinder proliferation of the pathogen. A similar phenomenon was reported by Norwood and Gilmour (2001) when they cultured *L. monocytogenes* in the presence of *Pseudomonas* and *Staphylococcus* and found their being in the same vicinity of the stainless steel coupons as *L. monocytogenes* to be one of the factors contributing to the decrease of *L. monocytogenes* cell numbers. The displacement attachment models used in the current study demonstrated that the LAB cocktails were even able to displace *L. monocytogenes* attached on the surface of coupons; however, the degree of pathogen reduction was lower than the responses observed for inhibition and competitive attachment. *L. monocytogenes* may have occupied the receptor areas on the coupons, and the LAB cocktails could have influenced the pathogen by inhibiting them access to nutrients available. When the LAB cocktails concentration was reduced from 10^8 to 10^6 CFU/ml, there was significant reduction ($P < 0.05$) of the pathogen only in the inhibition attachment model, which explains the enhanced inhibitory effect resulting from coating the surface with the LAB cocktails prior to *L. monocytogenes* addition. Additionally, this highlights the role of sequence of the LAB cocktails and *L. monocytogenes* treatment on the surface of the coupons in the inhibition of *L. monocytogenes*, which may be useful in designing systems to protect surfaces in the food industry.

Conclusions

Outbreaks linked to *L. monocytogenes* continue to occur, and the loss in terms of human life and economics associated with product recalls are substantial (Scallan et al., 2011). This study demonstrated that LAB reduced the attachment of *L. monocytogenes* to stainless steel. This capability can be used in combination with compounds such as sodium acetate, sodium diacetate or potassium sorbate previously characterized to inhibit the growth of *L. monocytogenes* in food (Lloyd et al., 2009), in multiple-hurdle technology geared to produce bio- sanitizers that prevent the attachment to and proliferation of *L. monocytogenes* on stainless steel surfaces.

Acknowledgment

Table 1: Total carbohydrate production of *L. monocytogenes*, three LAB strains and the combination of LAB strains in a cocktail in planktonic culture and after 3 hr attachment.

Strains	Planktonic (mg/log CFU) *	Attached (mg/log CFU) *
L. monocytogenes	0.64 ± 0.12 [A,a†]	0.69 ± 0.06 [A,a]
P. acidilactici	0.50 ± 0.03 [D,a]	0.60 ± 0.02 [A,b]
Lb. amylovorus	0.54 ± 0.04 [C,D,a]	0.68 ± 0.06 [A,b]
Lb. animalis	0.55 ± 0.03 [B,C,D,a]	0.69 ± 0.03 [A,b]
LAB cocktail	0.64 ± 0.05 [A,B,a]	0.67 ± 0.01 [A,a]

*Carbohydrate data are expressed as mean values ± standard deviation of three independent trials.

[†]Values with the different alphabetical notations within rows are statistically different ($P < 0.05$). Upper-case describes statistical difference of values within columns, whereas lower-case describes statistical difference of values within rows.

Table 2: Data of 24, 72 hr attachment of *L. monocytogenes* (10^3 CFU/ml), *P. acidilactici*, *Lb. amyvolorus*, *Lb animalis* (10^8 CFU/ml each), and the combination of the three strains (LAB cocktail, 10^8 CFU/ml) to stainless steel surface.

Strain	Counts (log CFU/cm^2) after 24 hr*	Counts (log CFU/ cm^2) after 72 hr*
L. monocytogenes	8.44 ±0.357 ^{C,a†}	9.67 ±0.378^{A,b}
P. acidilactici	8.60 ±0.63^{B,C,a}	8.85 ±0.40^{B,C,a}
Lb. amylovorus	4.03 ±3.0^{D,E,a}	3.79 ±2.99^{E,a}
Lb. animalis	6.44 ±0.79^{D,a}	8.33 ±0.97^{C,b}
LAB cocktail	8.60 ±0.48^{B,C,a}	9.09 ±0.33^{A,B,b}

*Attachment levels expressed as means values ± standard deviation of the mean of six independent trials.
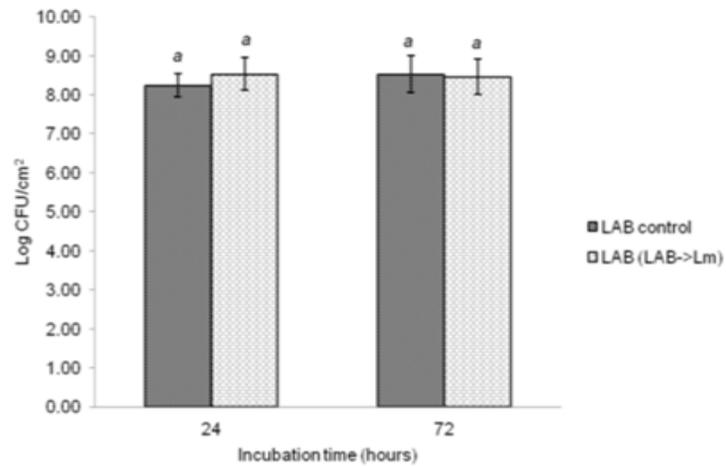
$\dagger$Values with different alphabetical notations are statistically different ($P < 0.05$). Upper-case describes statistical difference of values within columns, whereas lower-case describes statistical difference of values within rows.
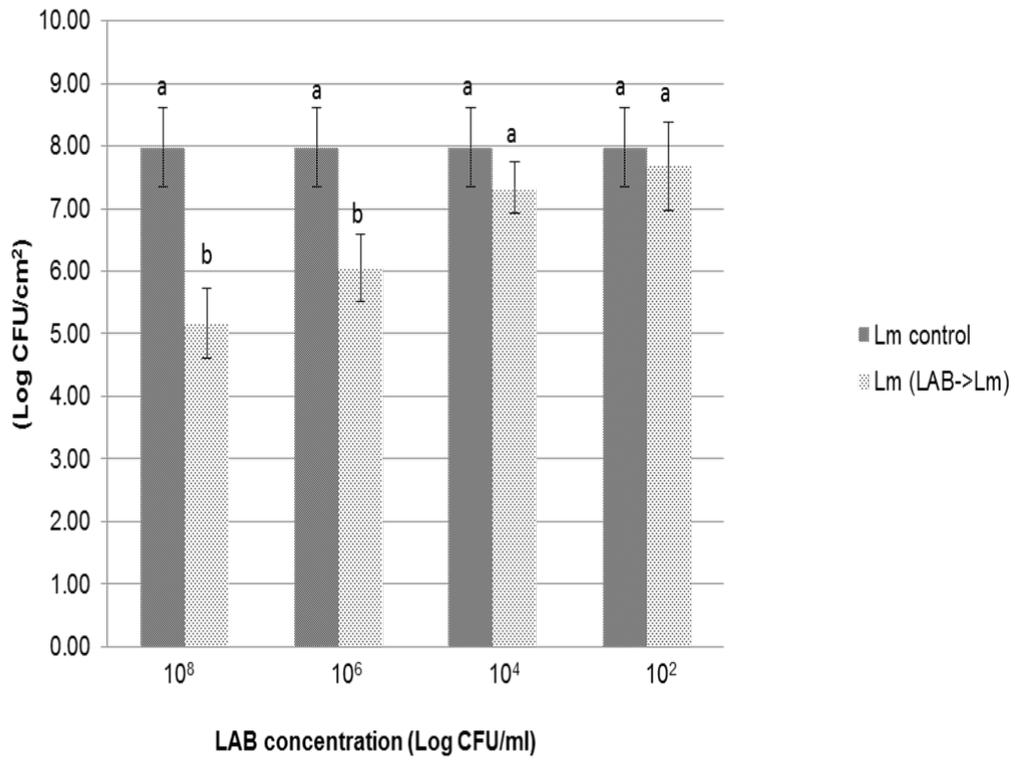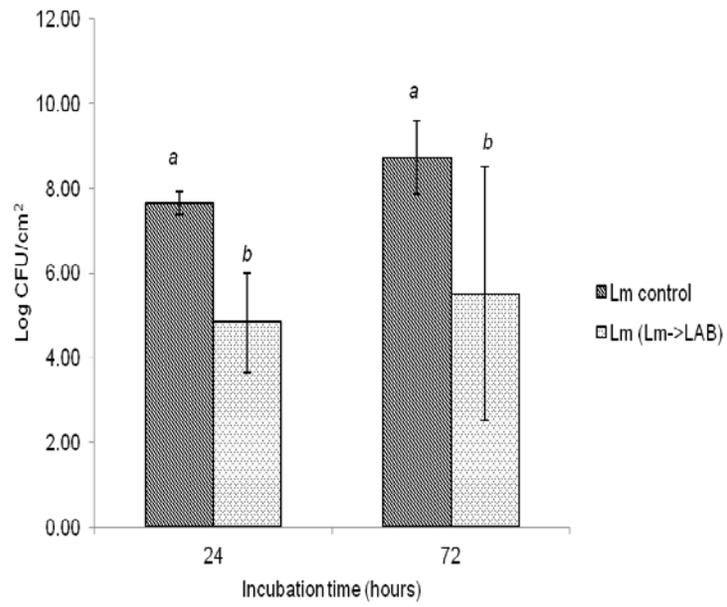
Figures

A)
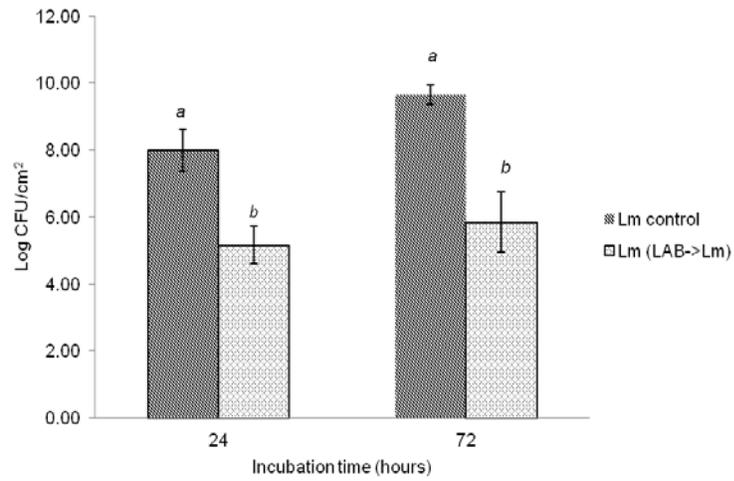
B)

C)

Figure 1: The inhibition of the attachment of *L. monocytogenes* (10^3 CFU/ml) by LAB (10^8 CFU/ml) on a stainless steel. LAB was allowed to attach to the coupons for 24 hr prior to the addition of *L. monocytogenes*. A) *L. monocytogenes* remained after 24 and 72 hr incubation. Lm (LAB $\rightarrow$ Lm): *L. monocytogenes* when the coupons were pre-treated with LAB for 24 hr. Incubation time is considered from the time after *L. monocytogenes* inoculation. B) LAB remained after 24 and 72 hr incubation. LAB (LAB $\rightarrow$ Lm): LAB when *L. monocytogenes* was added. C) Limit *of L. monocytogenes* attachment inhibition by different concentration of LAB after 24 hr. Lm (LAB $\rightarrow$ Lm): *L. monocytogenes* attached to the coupons when pre-treated with LAB (10^8, 10^6, 10^4, 10^2 CFU/ml). Different alphabetic notations indicate the significant difference ($P < 0.05$).
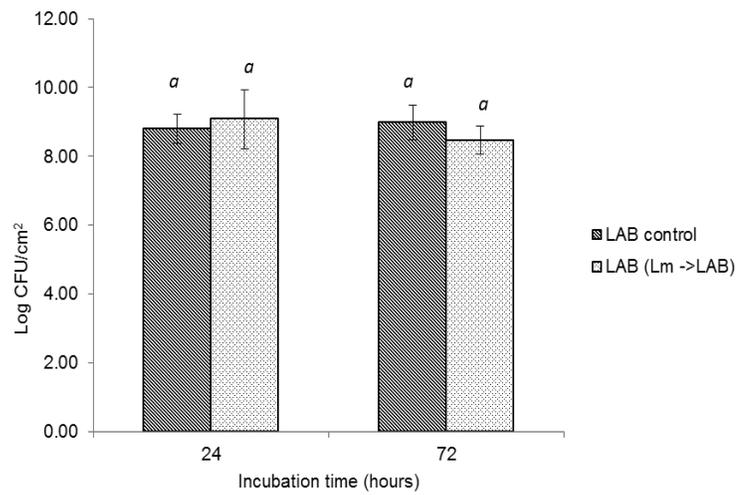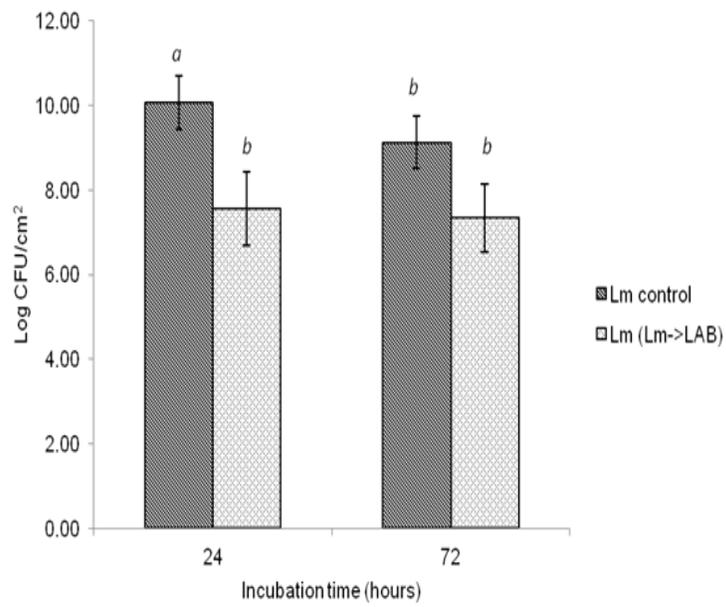
A)

B)

Figure 2: The competitive attachment inhibition between *L. monocytogenes* (10^3 CFU/ml) and LAB (10^8 CFU/ml) on stainless steel. A) *L. monocytogenes* remained after 24 and 72 hr incubation. Lm (Lm → LAB): *L. monocytogenes* remained when it was added to the coupons at the same time as LAB. Incubation time is considered from the time after inoculation of LAB and *L. monocytogenes* B) LAB remained after 24 and 72 hr incubation. LAB (LAB → Lm): LAB remained on the coupons when LAB and *L. monocytogenes* were added at the same time. Different alphabetic notations indicate the significant difference ($P < 0.05$).
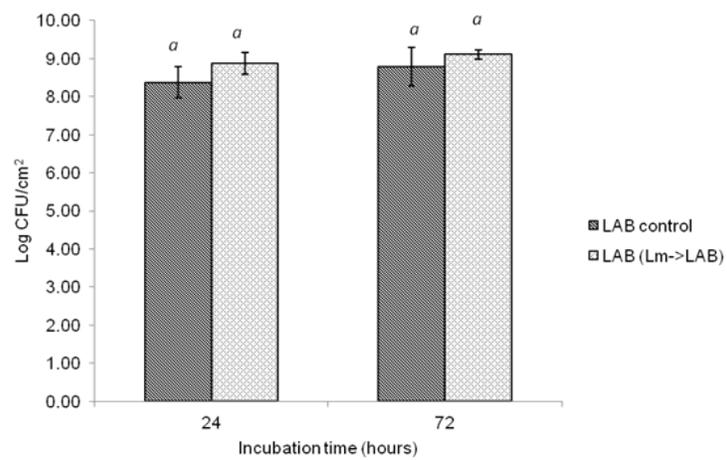
A)

B)

Figure 3: The displacement of *L. monocytogenes* (10^3 CFU/ml) by LAB (10^8 CFU/ml) on stainless steel. *L. monocytogenes* was pre-treated to the coupons for 24 hr prior to the addition of LAB cocktail. A) *L. monocytogenes* remained after 24 and 72 hr incubation. Lm (Lm → LAB): *L. monocytogenes* when the coupons were pre-treated with *L. monocytogenes* for 24 hr prior to the addition of LAB. Incubation time is considered from the time after LAB inoculation. B) LAB remained after 24 and 72 hr incubation. LAB (Lm → LAB): LAB when the coupons were pre-treated with *L. monocytogenes* 24 hr prior to the addition of LAB. Different alphabetic notations indicate the significant difference ($P < 0.05$).

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Influence of *Listeria innocua* on the attachment of *Listeria monocytogenes* on stainless steel and

aluminum surfaces

Influence of *Listeria innocua* on the attachment of *Listeria monocytogenes* to stainless steel and aluminum surfaces.

Jean Baptiste Ndahetuye, Ok Kyung Koo, Corliss A. O'Bryan, Steven C. Ricke, Philip G. Crandall (*).

Center for Food Safety, Department of Food Science, University of Arkansas, Fayetteville, 72704

Correspond author: Dr. Philip G. Crandall; Mailing address: Center for Food Safety, University of Arkansas, Fayetteville, AR 72704, e-mail: crandal@uark.edu.

Abstract

Listeria monocytogenes is an important foodborne pathogen that may be transmitted from the food-processing environment to food; however, the pathogen ecology and interaction with microbial resident on surfaces within the food industry is not well understood. The current study was undertaken to investigate the influence of *L. innocua* on the attachment of *L. monocytogenes* to stainless steel or aluminum at surfaces 23° C. The attachment was evaluated by placing the stainless steel and aluminum coupons in tryptic soy broth supplemented with 6 g/l yeast extract (TSB-YE). An aliquot containing 10^3 CFU/ml of both bacteria was deposited on the coupons, and the attached cells were quantified after 24 and 72 h. Cell populations in the mixed biofilms were lower by 0.3 log CFU/cm^2 than the levels of *L. monocytogenes* alone after 24 h ,whereas *L. monocytogenes* was higher by 0.7 log CFU/cm^2 than the counts of *L. innocua*, which indicate that *L. innocua* might have interfered with the attachment of *L. monocytogenes* to stainless steel surface. Similarly, there was a 0.5 log CFU/cm^2 difference observed between *L. monocytogenes* and the cell in mixed biofilms after 24 h on aluminum surface. There was a slight difference between treatments after 72 h contact time on both tested surfaces. *L. innocua* has an effect on the attachment of *L. monocytogenes*, increasing our understanding of the behavior of *L. monocytogenes* in the presence of another Listeria species. The results of the study may be used to tailor control strategies of *L. monocytogenes* biofilms.

Introduction

Listeria monocytogenes is an important food pathogen characterized by high mortality and hospitalization rates (Gandhi and Chikindas, 2007). It has been difficult to completely eliminate the pathogen from the food-processing premises because the organism is widely distributed in nature and can survive under extreme conditions of temperature, pH and salts (Cole et al., 1990, Gray and Killinger, 1966). Additionally, *L. monocytogenes* is able to attach to and form biofilms on materials found in the food-processing environment, and cells in biofilms develop resistance to cleaning and disinfection (Blackman and Frank, 1996; Beresford et al., 2001; Somers and Wong, 2004). *L. monocytogenes* in niches within processing facilities may cause contamination of food products (Lin et al., 2006; Wilks et al., 2006). Despite listeriosis outbreaks linking *L. monocytogenes* from the food- processing environment (CDC, 2011), investigation of the ecology of the pathogen and its interaction with other microbiota is not well understood (Hoelzer et al., 2011).

Interaction of microorganisms attached on surfaces may interfere with or promote *L. monocytogenes* biofilm formation and development. Norwood and Gilmour (2001) observed a decrease of *L. monocytogenes* cell numbers attached to stainless steel at 4° C, 18° C, and 30° C in presence of *Staphylococcus xylosus* and *Pseudomonas fragi*. The decrease was attributed to the competition for nutrients and production of antagonistic compounds by *S. Xylosus*. However, a separate study conducted by Sasahara and Zottola (1993) demonstrated that the attachment of *L. monocytogenes* in the mixed-cultures was enhanced by the exopolymer produced by *P. fragi* compared to the *L. monocytogenes* in pure culture (Sasahara and Zottola, 1993). The non-pathogen *L. innocua* is abundant in the environment and has been isolated in the same niches with *L. monocytogenes* such as ground water and surface water samples, or in fecal samples from

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the soil (Luppi et al., 1988; Weis and Seeliger, 1975; Wilshimer, 1968). Similarily, *L. innocua* and *L. monocytogenes* were isolated in rubber pipes of the cheese processing plants (Silva et al., 2003). Studying the influence of *L. innocua* on the survival of *L. monocytogenes* in the food-processing environment would further the understanding the ecology of *L. monocytogenes* in the food-processing environment.

Materials and methods

Bacterial strains

L. monocytogenes F6900 (serotype 1/2 a) (Cornell University, Ithaca, NY) and *L. innocua* ATCC 33090 (American Type Culture Collection) were used in the study. Both strains were from the culture collection at the Center for Food Safety, Department of Food Science, and University of Arkansas. Cultures were maintained in glycerol at - 80° C. Each strain was inoculated in tryptic soy broth supplemented with 0.6 % yeast extract (TSB-YE) (Becton, Dickinson & Co, Sparks, MD) and incubated at 37° C for 18 to 20 h before starting the experiment.

Coupon samples.

Stainless steel coupons (1.8 x 2.4 cm) cut from the blade of a Hobart Heavy deli slicer (Hobart Food Equipment, Australia) and aluminum coupons (2 x 2.5 x 0.5 cm) cut from the from the blade guard of the same slicer were used as a model of a food contact surface. The coupons were washed thoroughly with a dish detergent, rinsed well, wrapped in aluminum foil and autoclaved at 120° C for 15 min before use.

Attachment of the cells to the coupons

The method detailed by Norwood and Gilmour (2001) was adapted for bacterial attachment to stainless steel and aluminum. Treatments were (i) the coupons treated with *L. monocytogenes* (10^3 CFU/ml) (ii) the coupons treated with *L. innocua* (10^3 CFU/ml) (iii) the coupons treated with *L. monocytogenes* (10^3 CFU/ml) and *L. innocua* (10^3 CFU/ml). The coupons were placed into 6-well plates filled with TSB-YE. Each of the treatment was inoculated in the well of the plate. The plates were incubated at 23° C for 3 h to allow the bacteria to attach to the coupons. Thereafter, the coupons were washed carefully with 1 ml PBS three times in order to remove loosely bound cells. The coupons were placed in a fresh TSB-YE followed by incubation at 23° C for 24 and 72 h. After each incubation period, the coupons were aseptically recovered and rinsed with PBS three times. They were placed into tubes containing 15 ml PBS. Subsequently, the coupons were sonicated (Misonix Incorporated, Farmingdale, NY) in a bath for 3 min at 50 % amplitude to release bound cells. Appropriate dilutions were dispensed in duplicates on modified oxford agar (MOX) (EMD Chemical Inc., Gibbstown, NJ) plates for *L. monocytogenes* and *L. innocua* count. There were 3 independent trials and each trial had 2 replications.

Statistical analysis

One-way ANOVA analysis of variance tests were used to test differences among means for treatments at a significance level of 5 %. All calculations were performed using JMP statistical software (JMP Pro 9.02) (SAS Institute Inc., Cary, NC).

Results and Discussion

The attachment of *L. monocytogenes* on stainless steel at 23° C for 24 h was statistically not different from the attachment of *L. innocua* as well as the cell populations in biofilms formed

by the mixed bacteria; a similar trend was observed after 72 h. Nevertheless, cell populations in the mixed biofilm were lower by 0.3 log CFU/cm^2 than the level of *L. monocytogenes* after 24 h whereas *L. monocytogenes* was higher by 0.7 log CFU/cm^2 than *L. innocua* counts. After 72 h the difference between *L. monocytogenes* and cell populations in mixed biofilm leveled off; however, cells in mixed biofilms were still slightly lower than *L. monocytogenes* in pure biofilms.

Similarly, when aluminum was used as a surface of attachment, and the treatments were identical as in the previous setting, the attachment of *L. monocytogenes* was not statistically different ($P < 0.05$) from the cell populations in the mixed biofilm. However, 0.5 Log CFU/cm^2 difference was observed between *L. monocytogenes* and the cell population in mixed biofilms, and the difference leveled off at 72 h sampling time.

The results indicate that *L. innocua* influenced the attachment of *L. monocytogenes* on stainless steel and aluminum surfaces. *L. innocua* has been isolated in the same environmental niches as *L. monocytogenes* (Luppi et al., 1988), additionally Bourio and Cerf (1996) demonstrated that *L. innocua* is able enter and survive in *Pseudomas aeruginosa* biofilms in the food-processing environment, and is unable to survive alone (Bourion and Cerf, 1996). Furthermore, studies concluded that some *Pseudomonas* enhances the ability of *L. monocytogenes* biofilms (Hassan et al., 2004) whereas *S. sciuri* negatively effect *L. monocytogenes* attachment on stainless steel. To shed more light on the complexity of the ecology of *L. monocytogenes* on surfaces typically found in food-processing, the present study focused on evaluating the influence the non-pathogenic *L. innocua* on attachment of *L. monocytgenes* on stainless steel and aluminum surfaces. Cells in biofilms may compete for available nutrients and attachment site on surfaces resulting in a decrease of either species

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compared to their respective populations in pure biofilms (Norwood and Gilmour, 2001). *L. innocua* might have competed with *L. monocytogenes* the attachment sites available on stainless steel or aluminum, which resulted in the reduction of the later. Bacteria in biofilms are also reported to produce more exopolysaccharides than their free-living counterparts (Chae et al., 2006). Carbohydrates of one microorganism may play a role of anti-adhesive substances of the other thus hindering their development in biofilm (Leriche and Carpentier, 2000). Exoplysaccharide produced by either bacteria might have influence their levels of attachment. Real-time PCR would confirm the exact proportion of *L. monocytogenes* growing in the presence of *L. innocua*.

Conclusions

The current study tested the influence of *L. innocua* on the attachment of *L. monocytogenes* on stainless steel and aluminum surfaces. Although more tests are still needed, results indicate *L. innocua* to have a negative effect on the attachment of *L. monocytogenes*. Future research might focus on determining the exact proportion of *L. monocytogenes* and *L. innocua* in mixed biofilms and quantitative and qualitative evaluation of exopolymer produced by *L. innocua* and the impact they might have on the attachment of *L. monocytogenes*.
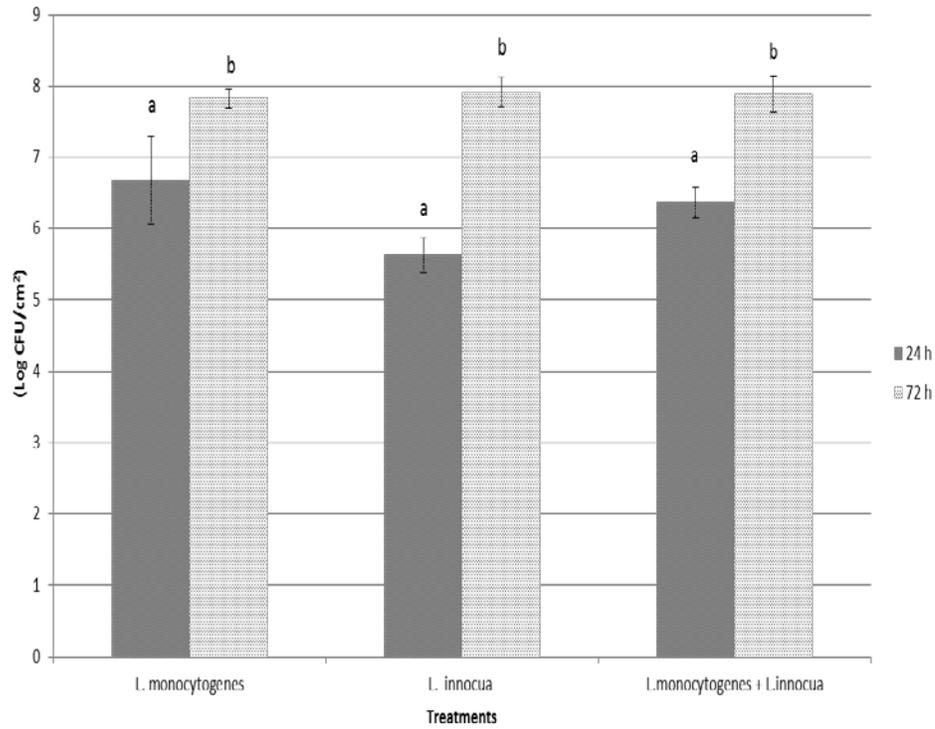
Figures

Figure 1: Attachment of *L. monocytogenes*, *L. innocua* and the mixed of both bacteria at the ratio 1:1 on stainless steel surface.
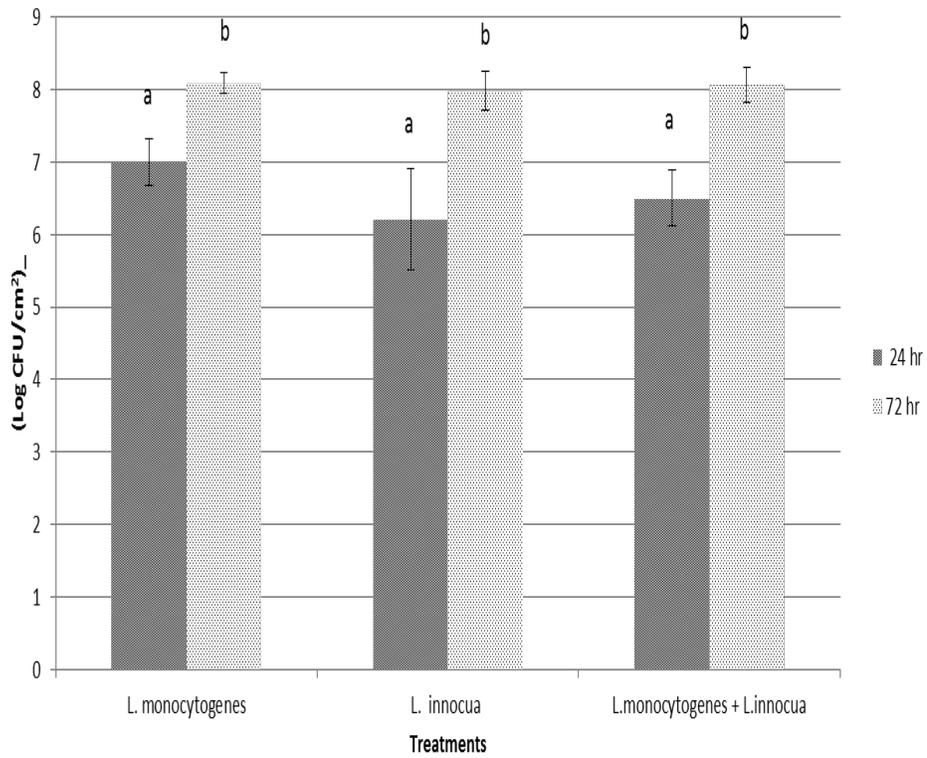
Figure 2: Attachment of *L. monocytogenes*, *L. innocua* and the mixed of both bacteria at the ratio 1:1 on aluminum.

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V.   Conclusions

The presence and adaptation of *L. monocytogenes* in the food-processing environment remains a serious concern especially in RTE food establishments. Lactic acid bacteria demonstrated the ability to attach and produce carbohydrates on stainless surface which are important characteristics for attachment. Antilisterial substances produced by LAB and competition of available nutrients and sites for attachment contributed to the decrease of the attachment of *L. monocytogenes* in presence LAB. This property may be used in manufacturing bio-sanitizer geared to control *L. monocytogenes* biofilms in food-processing environment settings. *L. innocua* also showed to have a negative effect on the attachment of *L. monocytogenes* on stainless steel and aluminum; however, additional tests are still needed to confirm the dynamics of interactions of both bacteria that resulted in interference of attachment of *L. monocytogenes*.