DANILO AUGUSTO LOPES DA SILVA

Listeria monocytogenes IN A BRAZILIAN PORK PRODUCTION CHAIN AND ADHESION FEATURES ISOLATES

Thesis presented to the Universidade Federal de Viçosa, as part of the requirements of the Graduate Program in Veterinary Medicine, to obtain the title of *Doctor Scientiae*.

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Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *Doctor Scientiae*.

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“Se acreditares em estrela, vai buscá-la." Fernando Sabino

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ABSTRACT

SILVA, Danilo Augusto Lopes da, D.Sc., Universidade Federal de Viçosa, July, 2019. ***Listeria monocytogenes* in a Brazilian Pork Production Chain and Adhesion Features of Isolates.** Adviser: Luís Augusto Nero. Co-advisor: Ricardo Seiti Yamatogi.

L. monocytogenes is present at low frequencies in animals to be slaughtered but may persist for long periods in the food processing environment. In the present study, *L. monocytogenes* contamination was evaluated at different stages of a pork meat production chain in the state of Minas Gerais. Ten lots of pigs were sampled at different stages of the production chain, covering samples from termination sheds, slaughtering (after bleeding, after singeing, after evisceration and after final washing), processing (knives, deboning tables and hand handlers) and end products (ribs, shoulder, ham and sausage) totaling 670 samples. All samples were submitted to *L. monocytogenes* detection, and the isolates obtained were characterized by biochemical analyzes, serogroups, virulence genes, PFGE, antibiotic susceptibility and adhesion ability. The results revealed the low occurrence of *Listeria* spp. in the pork production chain evaluated. However, four sausage samples tested (40%) were positive for *Listeria* spp., with *L. monocytogenes* identified in two (20%). Ten isolates were identified as *L. monocytogenes* (eight from serogrounp 1/2a or 3a and two from serogrounp 4b, 4d or 4e), all positive for virulence-related genes *hlyA*, *iap*, *plcA*, *actA*, *inlA*, *inlB*, *inlC* and *inlJ* and susceptible to the tested antibiotics. A sausage sample was contaminated by both serogroups 1/2a or 3a and 4b, 4d or 4e. Isolates from serogroup 1/2a or 3a obtained at visits 5 and 6 showed distinct genetic profiles by PFGE, suggesting that contamination may come from a different source. The adhesion potential exhibited by *Listeria* spp. isolates (n = 18) ranged from weak (serogroup 4b, 4d or 4e) to moderate (*L. innocua* and *L. monocytogenes* serogroup 1/2a or 3a). Despite the low occurrence of *L. monocytogenes*, pathogenic serogroups were detected in sausage, requiring industry control measures. Since *L. monocytogenes* is a pathogen capable of adhering to various surfaces and forming biofilms, which may explain its persistence in food processing environments, this work also evaluated *L. monocytogenes* adhesion capacity and the interference of stress factors on adhesion capacity of selected strains (*L. monocytogenes* strains belonging to lineages I and II, also coming from the meat processing environment, characterized in parallel work by strong adhesion potential (one isolate) and persistence capacity in the processing environment for 3 years (two isolates)), were incorporated into this work and tested with 3 selected isolates from sausage samples. These isolates were submitted to adhesion potential and minimum inhibitory concentration tests against four disinfectants. The adhesion capacity of the selected isolates was also tested considering: disinfectant dilutions, NaCl concentrations and curing salts, incubation time and temperature. Each isolate was classified according to its adherence capacity as weak, moderate or strong. The four disinfectants tested were effective in eliminating *L. monocytogenes*. The isolates selected for stress tests showed greater adhesion capacity at 37 °C/72 hours, and the BHI broth with 5% NaCl and quaternary ammonia (1: 1,024) were ineffective in inhibiting polystyrene adhesion. The collected data allowed the identification of the adhesion potential of *L. monocytogenes*, the efficacy of the sanitizers tested in the control of contamination by this pathogen and the adhesion capacity in the presence of quaternary ammonium (1: 1,024) and salts of some isolates. Considering their ability to adhere to stressful conditions in the tests described above and the fact that they have the genome sequenced by (cg) MLST in parallel studies four isolates of *L. monocytogenes* belonging to strains I and II from the meat processing environment were further investigated for stainless steel biofilm formation capacity in the presence of curing salt 7.5% (lineage I) and quaternary ammonium (1: 1,024) (lineage II). Additionally, a predictive analysis of gene expression related to biofilm formation and adaptation to stressful conditions was performed by qPCR assays (previously detected by *in silico* genome analysis of selected isolates, characterized by (cg) MLST in parallel work). *L. monocytogenes* biofilm formation in stainless steel was tested in two different systems (microplate and coupons) at 37 °C for 72 hours. Assays were performed as biological triplicates and included appropriate controls to verify significant differences by analysis of variance ($p < 0.05$). It was observed that the tested strains (I and II) were able to form stainless steel biofilm. Although the treatments used in each strain were significant in reducing the biofilm formation ($p < 0.05$), the isolates were able to form biofilm under the stress condition evaluated. *L. monocytogenes* biofilm suspensions from stainless steel coupon testing gave positive results in qPCR assays for eleven target genes tested. In general this work showed that the pig did not appear to be a representative carrier of this pathogen. Even though it was not possible to obtain positive samples for *L. monocytogenes* from the slaughtered and processing environment, obtaining final products (sausage) contaminated with pathogenic serogroups shows the health risk to the final consumer and the ability of this pathogen to persist in the pork meat processing environment once its presence has been detected in the final product. The isolates of *L. mococytogenes* obtained belong to phylogenetic lineages recognized for being highly adapted to the food processing environment, and showed ability to adhere to the tested surfaces under stress conditions. The recognized ability of this pathogen to form biofilm on surfaces commonly found in the meat processing environment could also be demonstrated, the tested isolates were able to form stainless steel biofilm in the presence of agents usually used for sausage preparations and also in the processing plant cleaning / disinfection procedures. The *in silico* analysis of cg MLST allowed the identification of genomic regions related to biofilm formation and adaptation to stressful environmental conditions in lineages I and II isolates. It is also possible to detect the action of this genetic machinery in the stainless steel biofilm formation under action of stress factors, by predicting the expression of eleven target genes performed by qPCR.

RESUMO

SILVA, Danilo Augusto Lopes da, D.Sc., Universidade Federal de Viçosa, julho de 2019. ***Listeria monocytogenes* em uma cadeia de produção de carne suína brasileira e características de adesão de isolados**. Orientador: Luís Augusto Nero. Coorientador: Ricardo Seiti Yamatogi.

L. monocytogenes está presente em baixas frequências em animais a serem abatidos, mas pode persistir por longos períodos no ambiente de processamento de alimentos. No presente estudo, a contaminação por *L. monocytogenes* foi avaliada em diferentes etapas de uma cadeia produtiva de carne suína no Estado de Minas Gerais. Dez lotes de suínos foram amostrados em diferentes etapas da cadeia de produção, abrangendo amostras de galpão de terminação, abate (após sangria, após chamuscamento, após evisceração e após lavagem final), processamento (facas, mesas de desossa e mão de manipuladores) e produtos finais (costelas, paleta, pernil e linguiça) totalizando 670 amostras. Todas as amostras foram submetidas à detecção de *L. monocytogenes*, e os isolados obtidos foram caracterizados por análises bioquímicas, sorogrupos, genes de virulência, PFGE, susceptibilidade à antibióticos e habilidade de adesão. Os resultados revelaram a baixa ocorrência de *Listeria* spp. na cadeia de produção de suínos avaliada. No entanto, quatro amostras de linguiça testadas (40%) foram positivas para *Listeria* spp., Sendo *L. monocytogenes* identificada em duas (20%). Dez isolados foram identificados como *L. monocytogenes* (oito de serogrounp 1/2a ou 3a e dois de sorogrupo 4b, 4d ou 4e), todos positivos para genes relacionados a virulência *hlyA*, *iap*, *plcA*, actA, inlA, *inlB*, *inlC* e *inlJ* e suscetíveis aos antibióticos testados. Uma amostra de linguiça foi contaminada por ambos os sorogrupos 1/2a ou 3a e 4b, 4d ou 4e. Os isolados do sorogrupo 1/2a ou 3a obtidos nas visitas 5 e 6 apresentaram perfis genéticos distintos por PFGE, sugerindo que a contaminação pode vir de fonte diferente. O potencial de adesão exibido por *Listeria* spp. isolados (n = 18) variaram de fraco (sorogrupo 4b, 4d ou 4e) a moderado (*L. innocua* e *L. monocytogenes* sorogrupo 1/2a ou 3a). Apesar da baixa ocorrência de *L. monocytogenes*, foram detectados sorogrupos patogênicos em linguiça, exigindo medidas de controle por parte da indústria. Uma vez que *L. monocytogenes* é um patógeno capaz de aderir a variadas superfícies e formar biofilmes, o que pode explicar sua persistência em ambientes de processamento de alimentos este trabalho também avaliou a capacidade de adesão de *L. monocytogenes* e a interferência de fatores estressantes na capacidade de adesão de cepas selecionadas (cepas de *L. monocytogenes* pertencentes às linhagens I e II, também provenientes do ambiente de processamento de carnes, caraterizadas em trabalhos paralelos por forte potencial adesão (1 isolado) e capacidade de persistência no ambiente de processamento por 3 anos (2 isolados)), estes isolados foram incorporados a este trabalho e testados junto a 3 isolados provenientes de linguiça. Estes isolados foram submetidos a testes de potencial de adesão e concentração inibitória mínima contra quatro desinfetantes. A capacidade de adesão dos isolados selecionados também foi testada considerando: diluições de desinfetantes, concentrações de NaCl e sais de cura, tempo de incubação e temperatura. Cada isolado foi classificado de acordo com sua capacidade de adesão como fraco, moderado ou forte. Os quatro desinfetantes testados foram eficazes na eliminação de *L. monocytogenes*. Os isolados selecionados para testes de estresse apresentaram maior capacidade de adesão a 37 °C/ 72 horas, e os meios com 5% de NaCl e amônia quaternária (1:1,024) foram ineficazes na inibição da adesão ao poliestireno. Os dados coletados permitiram a identificação do potencial de adesão de *L. monocytogenes*, da eficácia dos sanitizantes testados no controle da contaminação por este patógeno e a capacidade de adesão na presença de amônio quaternário (1:1024) e sais de alguns isolados. Considerando sua habilidade de adesão em condições estressantes nos testes descritos acima e o fato de terem o genoma sequênciado por (*cg*) MLST em estudos paralelos quatro isolados de *L. monocytogenes* pertencentes às linhagens I e II provenientes do ambiente de processamento de carne foram ainda investigados para capacidade de formação de biofilme em aço inoxidável na presença de sal de cura 7.5% (linhagem I) e amônio quaternário (1:1,024) (linhagem II). Adicionalmente foi realizada uma análise preditiva da expressão dos genes relacionados à formação de biofilme e adaptação a condições estressantes por ensaios qPCR (previamente detectados por análise *in silico* do genoma dos isolados selecionados, caracterizado por *cg* MLST em trabalhos paralelos). A formação de biofilme de *L. monocytogenes* em aço inoxidável foi testada em dois sistemas diferentes (microplaca e cupons) a 37 °C por 72 horas. Os ensaios foram realizados como triplicatas biológicas e incluíram os controles apropriados para verificar diferenças significativas pela análise de variância ($p < 0,05$). Foi possível observar que as linhagens testadas (I e II) foram capazes de formar biofilme em aço inoxidável. Embora os tratamentos utilizados em cada linhagem tenham sido significativos na redução do biofilme formado ($p < 0,05$), os isolados foram capazes de formar biofilme na condição de estresse avaliada. As suspensões de biofilme de *L. monocytogenes* a partir de testes em cupons de aço inoxidável deram resultados positivos em ensaios de qPCR para onze genes alvo testados. De maneira geral este trabalho demonstrou que o suíno não pareceu ser um portador representativo deste patógeno. Mesmo que não tenha sido possível a obtenção de amostras positivas para *L.*

monocytogenes oriundas do ambiente de abate e processamento amostrado, a obtenção de produtos finais (linguiça) contaminados com sorogrupos patogênicos evidencia o risco de saúde ao consumidor final e a capacidade deste patógeno em persistir no ambiente de processamento de carne suína, uma vez que sua presença foi detectada no produto final. Os isolados de *L. mococytogenes* obtidos pertencem às linhagens filogêneticas reconhecidas por serem altamente adptadas ao ambiente de processamento de alimentos, e apresentaram habilidade em aderir às superfícies testadas sob condições de estresse. A reconhecida capacidade deste patógeno em formar biofilme em superfícies comumente encontradas no ambiente de processamento de carnes também pôde ser demonstrada, os isolados testados foram capazes de formar biofilme em aço inoxidável na presença de agentes usualmente utilizados para preparações de embutidos e também nos procedimentos de limpeza e desinfecção da planta de processamento. A analise *in silico* do cg MLST destes isolados permitiu a identificação de regiões genômicas relacionadas à formação de biofilme e adaptação a condições ambientais estrassantes, em isolados das linhagens I e II, sendo ainda possível detectar a ação desta maquinaria genética na formação de biofilme em aço inoxidável sob ação de agentes estressantes, pela predição da expressão de onze gene alvo realizada por qPCR.

INTRODUCTION

L. monocytogenes is a foodborn pathogen that is frequently isolated in food industries (BERSOT *et al.*, 2008), and it is an ubiquitous microorganism highly adapted to survive in the environment (FENLON *et al.*, 1999). The ability to withstand stress conditions and form biofilms allows *L. monocytogenes* to persist in meat processing industries, causing health concerns. Studies have demonstrated the importance of *L. monocytogenes* as an environmental contaminant in pig slaughtering or processing industries in Brazil (BARROS *et al.*, 2007; BERSOT *et al.*, 2008).

Usually, this pathogen is present at low frequencies in animals to be slaughtered, but they persist for long periods in utensils and equipment used in the processing environment, which represent constant sources of contamination to meat products produced from pork or other (BARROS *et al.*, 2007). In this context, ready-to-eat pork produced play an important epidemiological role in the transmission of *L. monocytogenes* to humans, as during the different stages of processing, the pathogen can easily contaminate the handled products which will not suffer additional heat treatment until consumption (MARTINS; GERMANO, 2011). These evidences demonstrate the importance of controlling *L. monocytogenes* during the different stages of pork processing, aiming to minimize the contamination to final products.

In recent years, the prevalence of *L. monocytogenes* in food products has shown an upward trend (YU; JIANG, 2014). Its wide distribution in the environment is one of the main reasons, of this pathogen being a causative agente of food poisoning. Although human listeriosis is considered a disease with sporadic occurrence (FARBER; PETERKIN, 1991), several outbreaks have been observed in the last decades. This is because foodborne transmission is the main route of acquisition of listeriosis (SCHUCHAT; SWAMINATHAN; BROOME, 1991).

Although antibiotic therapy is effective in most cases, listeriosis is a serious public health problem, as it is fatal in up to 30% of cases (JONES; MACGOWAN, 1995). In general, *L. monocytogenes* isolates, as well as *Listeria* spp. strains are susceptible to a wide range of antibiotics, except cephalosporins and fosfomycin (SAFDAR; ARMSTRONG, 2003).

Unregulated use of antimicrobials in animals and humans may select resistant bacterial populations. In animal feed, for example, antibiotics are used for the control and treatment of infectious bacterial diseases, as well as for growth promotion (PHILLIPS *et al.*, 2004). In addition, chromosomal mutations and lack of selective pressure may have contributed to the spread of resistant bacteria in foods (CONTER *et al.*, 2009). *Listeria* spp. can acquire antibiotic resistance genes through plasmids and transposons from other bacterial species, either in *in vitro* or *in vivo* studies (POURSHABAN *et al.*, 2002). The study of antimicrobial susceptibility is important because it provides the identification of the emergence of resistant strains and provides useful information for the development of public health policies in the use of antibiotics, what can be used as phenotypic epidemiological markers (VAZ, 2008).

Meat can be contaminated from a wide variety of sources, because *L. monocytogenes* is widely distributed in the environment, in food processing plants, in homes, in agricultural settings, and in healthy humans and animals (LÓPEZ, VICTORIA *et al.*, 2008). Contamination of meat products in processing plants mostly appears to be due to *L. monocytogenes* strains already present in the plant environment, with the processing environment serving as the source of contamination. The contamination level has been observed to increase along the processing line. To reduce the contamination, processing plants should adhere to specific microbiological control measures, and cleaning and disinfection procedures should be strengthened (LÓPEZ, VICTORIA *et al.*, 2008)

The use of disinfectants has been incorporated into good production practice procedures to avoid the accumulation of microbial cells and consequent biofilm formation (HOOD; ZOTTOLA, 1995). However, several disinfectants widely used in the food processing environment may not be effective against some bacterial biofilms (RYU; BEUCHAT, 2005) and some alternative removal strategies are being studied. This work also analyzed the effectiveness of the main sanitizers used in the meat processing environment against *L. monocytogenes* strains, as well as evaluated the interference of stress factors (presence of salts, disinfectants and temperature) in the induction of the biofilm in stainless steel. Finally, the biofilm suspensions obtained in the above-mentioned tests were analyzed by qPCR for detection of gene expression related to biofilm formation and adaptation to environmental stress.

LITERATURE REVIEW

Listeria monocytogenes has been recovered from a variety of foods. Raw and processed meats, soft cheeses, raw milk, hot dogs, seafood and fresh vegetables have been associated with sporadic cases and outbreaks of listeriosis (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006). This pathogen is one of the main concerns of the meat industry, and outbreaks of listeriosis have been reported in the United States and Europe (ANDRITSOS *et al.*, 2013).

Pork and processed pork products, such as cold meats, were implicated in outbreaks of listeriosis in France and other European countries during the 1990s. This pathogen is particularly a pre-consumption in raw, undercooked and especially ready-to-eat foods for consumption (RTE). As the micro-organism is ubiquitous, food processing industries are easily contaminated by raw foods (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b).

Although the most recent outbreaks of Listeriosis related to the consumption of RTE foods are not associated with the consumption of meat-based RTE products and more specifically RTE derived from pork, important studies around the world have evaluated the prevalence and persistence of contamination by *L. monocytogenes* in meat based RTE products and pork products, mainly sausages (Table 1). These studies evidenced the presence of pathogenic strains belonging to serogroups capable of determining disease in humans. Some studies were able to relate strains of *L. monocytogenes* from severe cases of hospital admission with the consumption of meat based RTE products.

Studies have demonstrated that recontamination can be considered as the main source of *L. monocytogenes* in many commercially prepared processed foods (TOMPKIN, 2002). This finding led to significant changes in the way that the post-processing environment is managed. Modifications in the layout of the plant and equipment design, procedures for cleaning and hygienic practices and personal care were required to avoid *L. monocytogenes* contamination. It is expected that *L. monocytogenes* will continue to be introduced into the environment in which RTE foods are exposed for further processing and packaging (TOMPKIN, 2002).

By controlling the establishment and growth of *L. monocytogenes* in these environments, it is possible to minimize (and in some cases to prevent) the risk of contamination of the product with sanitation procedures (TOMPKIN, 2002). Depending on the food and the environmental control program, it should be possible in most food processes to include a validated listericide step (eg: cooking) thereby maintaining the product contamination prevalence at < 0.5%. If that goal can be achieved by assuming random distribution at a product contamination level of 0.5%, then there would be a 61% probability that a production batch would be accepted, even if 100 samples had been tested. Thus, final product tests become of little value for evaluation and verification control (TOMPKIN, 2002). Another disadvantage of product testing is that if a product is considered positive, no information obtained will indicate how the contamination was or how to avoid other occurrences.

Ideally, the control of contamination of *L. monocytogenes* is improved by monitoring the processing environment, instead of checking this foodborne pathogen only in end products. Based on data collected in the processing environment, it is possible to identify potential trends that can indicate a potential loss of control, allowing corrective procedures. It has been described in some environmental samples that "most of the strains are pathogenic, some strains may be pathogenic, some strains are not pathogenic" (HOF; NICHTERLEIN; KRETSCHMAR, 1994; MOURA *et al.*, 2016), and another study comparing different methods to evaluate virulence, demonstrated in a plaque formation assay using a monolayer of HT-29 cells three other classifications: virulent, hypovirulent and completely virulent (ROCHE *et al.*, 2001).

L. monocytogenes virulence is basically influenced by six genes in the cluster chromosome of virulence of PrfA-dependent genes and other genes important in virulence (for example, genes from the group of internalins) located outside this gene cluster. Presumably, strains with a total complement of virulence genes would have greater potential to cause disease, but the prevalence of such strains in food processing environments is not known. There is also speculation that certain strains have high potential to survive under adverse conditions and to multiply in the processing environment and/or in certain foods (MAURY *et al.*, 2019; TOMPKIN, 2002).

Worldwide, three serotypes (4b, 1/2a, and 1/2b) account for 89-96% of cases of human listeriosis, providing additional evidence that certain strains are more likely to cause disease. More interesting is the perception that a small number of clonal lineages have been responsible for large outbreaks documented in different regions of the world (MAURY *et al.*, 2016; NIELSEN *et al.*, 2017).

Table 1. Studies related to the research of *L. monocytogenes* contamination in meat based RTE products and pork products specifically.

Year	Location	Product	Estudo
2000	UK	meat based RTE product	(GILLESPIE; LITTLE; MITCHELL, 2000)
2002	Switzerland	meat based RTE product	(JEMMI; PAK; SALMAN, 2002)
2002	Brazil	meat based RTE product	(CURVELO *et al.*, 2002)
2005	Turkey	meat based RTE product	(ALIŞARLI; ATASEVER; GÖKMEN, 2005)
2006	Greece	meat based RTE product	(ANGELIDIS; KOUTSOUMANIS, 2006)
2009	Germany	meat based RTE product	(NETSCHAJEW *et al.*, 2009)
2009	Lithuania	meat based RTE product	(BERZIŅS; TERENTJEVA; KORKEALA, 2009)
2010	Spain	meat based RTE product	(PÉREZ-RODRÍGUEZ *et al.*, 2010)
2012	Germany	meat based RTE product	(MEYER *et al.*, 2012)
2015	Chile	meat based RTE product	(SALUDES; TRONCOSO; FIGUEROA, 2015)
2000	Europe	RTE sausage /salami	(LÓPEZ, M *et al.*, 2000)
2001	Greece	RTE sausage /salami	(METAXOPOULOS; SAMELIS; PAPADELLI, 2001)
2003	Brazil	RTE sausage /salami	(ICHIRO SAKATE *et al.*, 2003)
2005	France	RTE sausage /salami	(THÉVENOT *et al.*, 2005b)
2006	Italy	RTE sausage /salami	(GIANFRANCESCHI *et al.*, 2006)
2006	Ireland	RTE sausage /salami	(OZBEY; ERTAS; KOK, 2006)
2007	Latvia	pork meat based RTE product	(BERZINS *et al.*, 2007)
2007	Turkey	RTE sausage /salami	(COLAK *et al.*, 2007)
2007	France	RTE sausage /salami	(LEBERT *et al.*, 2007)
2007	Italy	RTE sausage /salami	(DE CESARE; MIONI; MANFREDA, 2007)
2007	Brazil	pork meat based RTE product	(DEGENHARDT; SANT'ANNA, 2007)
2009	Bulgaria	pork meat based RTE product	(KARAKOLEV, 2009)
2010	Italy	pork meat based RTE product	(PETRUZZELLI *et al.*, 2010)
2011	Brazil	pork meat based RTE product	(MARTINS; GERMANO, 2011)
2011	Brazil	pork meat based RTE product	(FAI *et al.*, 2011)
2013	Greece	pork meat based RTE product	(ANDRITSOS *et al.*, 2013)
2015	Greece	RTE sausage /salami	(MELONI, 2015)
2016	China	pork meat based RTE product	(LI *et al.*, 2016)
2017	France	pork meat based RTE product	(TRIMOULINARD *et al.*, 2017)
2017	Turkey	RTE sausage /salami	(OZBEY; OZBEY; KOK, 2017)
2017	Denmark	pork meat based RTE product	(MARTÍN; BOVER-CID; AYMERICH, 2017)
2018	France	pork meat based RTE product	(FÉLIX *et al.*, 2018)
2019	Brazil	pork meat products	(SERENO *et al.*, 2019)

RTE: ready-to-eat

In summary, the information obtained so far indicates that certain strains of *L. monocytogenes* are more virulent and much more likely to be involved in foodborne diseases than others. This information may help food processors understand why foods from certain establishments and not from other establishments have been implicated as a source of listeriosis, even though there are comparable rates of contamination between the establishments in question. Virulence is only an important factor involved in the complex events that lead to disease, and should be taken into account in the development of strategies for *L. monocytogenes* control (TOMPKIN, 2002).

1 Raw meat contamination and pork meat processing environments

Over the years *L. monocytogenes* has been isolated from raw pork and although the source of the contamination is not clearly defined, this pathogen has been occasionally isolated on farms in the feces and skin of apparently healthy pigs (NØRRUNG; ANDERSEN; SCHLUNDT, 1999). The microorganism is believed to be sheltered in the intestinal tract, the first studies revealed a prevalence of *L. monocytogenes* in swine fecal samples ranging from 0 to 47%, and high prevalences were reported in Eastern Europe in the 1990s (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b). Animal husbandry practices involving feeding dry animals or silage, breeding on closed farms and maintaining pathogen-free herds were considered in the variation of *L. monocytogenes* incidence in healthy pigs (FENLON; WILSON; DONACHIE, 1996).

There is also an understanding that carcasses are contaminated when the large intestine is ruptured during evisceration. However, there have been reports that *L. monocytogenes* was detected in pig carcasses, but not in the rectal contents of these animals prior to slaught (KANUGANTI *et al.*, 2002b), this may occur because the large slaughter plants have the practice of to evacuate the rectum before evisceration, minimizing rectal contents and consequently contamination of the subsequent carcass (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b).

Some authors have suggested that not all isolates of *L. monocytogenes* detected in carcasses have fecal origin, demonstrating that porcine carcasses were more likely to harbor *L. monocytogenes* in tonsils than intestinal contents (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b). Autio *et al.*, (2000) also reported the presence of *L. monocytogenes* in tongues and tonsils sampled in slaughterhouses. Interestingly, Kanuganti *et* *al.* (2002) showed that *L. monocytogenes* was detected more frequently in tonsil homogenates sampled in slaughterhouses when compared to the smears of tonsils collected inthe farm.

Studies have shown that the prevalence of *L. monocytogenes* in tonsils ranged from 0 to 61%, and this range is probably due to differences in sampling techniques and/or agricultural management methods (FENLON; WILSON; DONACHIE, 1996). Autio *et al.*, (2000) found that the occurrence of *L. monocytogenes* differed among refrigerators. In addition, the level of contamination of viscera (tongue, esophagus, trachea, lungs, heart, diaphragm, kidneys and liver) was particularly high (64%), and it was hypothesized that *L. monocytogenes* spreads through contact between the tonsils and tongue with other viscera and carcasses during the evisceration process.

Kanuganti *et al.*, (2002a) showed the frequency of *L. monocytogenes* contamination was lower in tissues of freshly slaughtered pigs and also in the small intestine when compared to ground pork. In addition, an extensive study of meat contamination levels in the industry (meat processing) indicated that the refrigeration and deboning steps significantly increased contamination of pork (NESBAKKEN; KAPPERUD; CAUGANT, 1996).Van den Elzen *et al.*, (1993) reported a high environmental prevalence of the pathogen in the areas of cooling (71 to 100%).

These results strongly suggest that the actions in the post-processing stage of slaughtering are a significant cause of contamination of the meat and that contamination becomes highin the areas of the refrigeration room and cutting environments (NESBAKKEN; KAPPERUD; CAUGANT, 1996). PFGE pulsed field gel electrophoresis studies, which characterized *Listeria* strains with origins related to raw meat and cutting surfaces during meat processing, have conclusively demonstrated that raw meat can contaminate meat processing environments. Conversely, contaminated equipment may in turn contaminate the by-products during processing (THÉVENOT *et al.*, 2005a).

Meat in general is mainly contaminated with *L. monocytogenes* serotypes 1/2a, 1/2b and 1/2c (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b), and this is also true for raw pork. Most cases of listeriosis are sporadic and often involve serotypes 1/2a and 1/2b (SCHUCHAT; SWAMINATHAN; BROOME, 1991). The presence and prevalence of serotype 1/2a, and to a lesser extent serotype 1/2b, in meat processing facilities may be a source of sporadic cases.

Thevenot *et al.*, (2005) reported the presence of serotype 4b strains in raw pork meat in a French pork industry. On the other hand, Giovannacci *et al.*, (1999) did not find this serotype in the slaughter of pigs and cutting plants. However, the presence of this serotype in pork is of great importance for public health, since serotype 4b is responsible for most of the outbreaks of listeriosis in France (CHARLIER *et al.*, 2017; GOULET *et al.*, 1998).

In general, the main source of food contamination by *L. monocytogenes* prior to release to consumers appears to be the processing environment. Several studies have shown that strains of *L. monocytogenes* isolated from meat processing environments are often serotypes 1/2a, 1/2b and 1/2c. Serotypes 4b and 4e were also isolated in pork processing industries in France (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006).

Different molecular techniques (AFLP, PFGE, PCR and ribotyping) showed that genotypes of *L. monocytogenes* strains collected in pork processing environments are diverse. The high genotypic diversity between strains collected from both surfaces during processing and from processed products indicate a continuous source of inoculation by raw material or the persistence of several strains, despite the cleaning and disinfection operations being applied. *L. monocytogenes* strains were found to be both transient (sporadic) and resident (persistent) strains (LUNDÉN *et al.*, 2003; THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b).

L. monocytogenes can settle in the processing environment and survive for a long period of time. There are reports of the persistence of strains over a year in two pork processing plants and for 3 years in a meat grinder (AUTIO, TIINA *et al.*, 2002). This pathogen adheres to the surfaces found in food processing environments, although there are differences in both extent and rate of adsorption according to surface type, pre-treatment, environmental conditions and bacterial serotypes (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b). Biofilms develop as a result of adsorption, adhesion of free floating cells and continuous growth of cells in the biofilm matrix.

L. monocytogenes can incorporate into a biofilm, although not all strains have the same ability to evolve into a mature biofilm (CHAE; SCHRAFT, 2000). Some authors have observed that the rate of cell multiplication of floating *L. monocytogenes* versus the biofilm cell forms was different and suggested that the growth behavior differed according to the carrier medium.

Kalmokoff *et al.*, (2001) found few differences in the adsorption rates of different *L. monocytogenes* strains, but significant differences in their adhesion and ability to form a mature biofilm were observed. Highly adherent strains produced fibrils that were generally absent in low adherence strains (VATANYOOPAISARN *et al.*, 2000).

An analysis that examined 111 strains of *L. monocytogenes* it was observed that strains belonging to serotype 1/2c adhered more significantly to stainless steel when compared to other serotypes over a 24-hour period (NORWOOD; GILMOUR, 2000). Autio *et al.*, (2000) also noted differences in short-term adherence of resident and non-resident strains on a stainless steel model surface, and serotype 1/2c strains also demonstrated a higher degree of adsorption.

A study led by Peccio *et al.*, (2003) that isolated *L. monocytogenes* from 11 food processing plants revealed that certain genotypes were more likely to cause (persistent) contamination of food facilities. These authors hypothesized that resident strains adapt to food processing facilities via selection. However, Lundén *et al.*, (2003) observed several strains of *L. monocytogenes* with similar PFGE profiles that were categorized as persistent in one plant but not in another, emphasizing the complex nature of persistence and non-persistent contamination. The authors suggested that, as additional samples were collected, some of the non-persistent strains of *L. monocytogenes* could have been recovered more frequently and were categorized as persistent.

2 Processed meat products and pork processing industry

Processed meat products may be contaminated by *L. monocytogenes* in several steps during their production: first, the raw materials may be contaminated and the manufacturing process is insufficient to maintain the product sterile or by contact with contaminated unprocessed raw materials and surfaces or people contaminated (CHASSEIGNAUX *et al.*, 2001).

People are known routes of contamination by *L. monocytogenes* (REIJ; DEN AANTREKKER; FORCE, 2004). Poor hygiene practices by workers, including simple procedures such as hand washing, were identified as the cause of pathogen transmission (AFSSA 2000). Cross-contamination can occur at any stage between the meat processing plant and the consumer's home (REIJ; DEN AANTREKKER; FORCE, 2004). Household contamination often occurs when the same cooking utensils, like cutting boards, are used in food containing contaminated raw materials and then used in other foods (cooked or ready for consumption) (AFSSA, 2000). There has been sporadic information on the occurrence of *L. monocytogenes* in processed pork meat products. First, because contamination may occur after manufacturing, in the home kitchen (AFSSA, 2000). Secondly, many meat products are speciality items which are region or country specific (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b).

However, many studies have documented the prevalence of *L. monocytogenes* contamination in a variety of pork products. *L. monocytogenes* was isolated in this category of products in Japan and countries of Europe, North and South America (DUFFY *et al.*, 2001; INOUE *et al.*, 2000; THÉVENOT *et al.*, 2005b). As with raw pork, strains of serotypes 1/2a, 1/2c and 1/2b are more common in processed pork products (JAY, 1996), while serotype 4b is less related (GREENWOOD; ROBERTS; BURDEN, 1991). However, strains with this serotype have been isolated from pork products (MARTINEZ *et al.*, 2003; THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b). The presence of 4b strains in the meat processing industry may result in sporadic cases or an outbreak of listeriosis, especially if foodstuffs are consumed by people from the at-risk population.

The presence of strains sharing identical pulses in raw meat, meat processing environment and final products is an evidence that certain clones have adapted to the processing environment of meat and processed products (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b). Chasseignaux *et al.* (2001) observed the same profiles in several plants that were from different geographic areas. Autio *et al.* (2002) also observed that certain strains were repeatedly recovered from pork products from several unrelated plants, indicating that these strains are not always plant-specific. Certain strains may be more widely distributed in nature and thus more easily (re) introduced into processing plants via raw products.

L. monocytogenes was isolated from a wide range of pork products (AFSSA, 2000; FICT, 2002). The growth and survival of a microorganism depend on its ability to overcome the environmental obstacles encountered during manufacturing and preservation processes such as temperature, pH, water activity, ions and nutrient availability. Most bacteria, including *L. monocytogenes*, are usually resistant to small changes in a specific environmental parameter, but severe or multiple changes stimulate complex stress responses that are usually directed at survival rather than multiplication (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b). The various environmental obstacles that are used by the food processing industry are discussed below.

3 Food-mediated barriers to the growth of *L. monocytogenes*

Temperature is the most important obstacle encountered by *L. monocytogenes*. This pathogen is capable of multiplying in temperature intervals from 0 to 45°C, while the optimum temperature is between 30 and 37 °C (AFSSA, 2000). It is capable of multiplying in refrigerated foods and is of great importance for food risk assessment even when initial contamination is low, the micro-organism can multiply during refrigeration and reach levels up to 100 CFU/g (AFSSA, 2000).

The temperature of the domestic refrigerators is often closer to 9 °C instead of 4 °C (SERGELIDIS *et al.*, 1997), which also favors the multiplication of *L. monocytogenes*. Fleming *et al.*, (1985) have shown that the thermotolerance of *L. monocytogenes* is higher than many other foodborne pathogens. Its heat-tolerance may increase after exposure to a variety of stress conditions, including heating in sublethal temperatures or osmotic and acid shocks. Linton *et al.*, (1992) observed that the effect of a heat shock on *L. monocytogenes* was highly dependent on temperature and duration of treatment: high temperatures and long time treatments increase resistance. Intrinsic factors to underlying foods also affect the influence of thermotolerance. Pork products that are cooked at very high temperatures are most likely free of *L. monocytogenes* cells (AFSSA, 2000).

There are reports of viable strains of *L. monocytogenes* at pH levels between 4.6 to 9 with an optimal pH between 7 (AFSSA, 2000). This pathogen may be subject to low pH levels during the processing of fermented varieties, such as dried and cured sausages (salamis, chorizo) (THÉVENOT *et al.*, 2005a). It is also capable of surviving highly acidic pH, characteristic of human stomachs and macrophagic phagosomes. This characteristic can be considered as a virulence factor because many other bacteria are not able to survive under these conditions (COTTER; HILL, 2003).

Glutamate decarboxylase (GAD), which modulates the pH of the intracellular system in certain Gram-positive cells, is considered the key mechanism by which *L. monocytogenes* maintains pH homeostasis in acidic environments. Strains that exhibit reduced GAD activity are more sensitive to gastric fluid; in addition, GAD activity is significantly correlated with acid tolerance in this organism. The GAD system plays a role in acid tolerance during the logarithmic and stationary growth phases and it is necessary for the induction of an optimal acid tolerance response (COTTER; HILL, 2003). However Vialette *et al.*, (2003) tested the pH sensitivity in strains of *L. monocytogenes*, which were isolated from meat products and food processing environments and they were generally more sensitive in the exponential multiplication phase compared to stationary phase.

The normal pH of the meat (5 to 5.5) can induce a tolerance response to the acid pH in *L. monocytogenes*, which in turn decreases the sensitivity of the organism to a low pH (FALEIRO; ANDREW; POWER, 2003). Several authors have noted that acid adaptation may induce cross-protection against several other deleterious factors, including osmotic stress. This cross-stress protection is strain dependent. The molecular processes involved have not yet been identified. Finally, sub-lethal stressing factors, such as osmolarity, heat and low temperatures, do not seem to affect the acid resistance of this bacterial species (KOUTSOUMANIS; KENDALL; SOFOS, 2003).

L. monocytogenes can be challenged to cope with saline concentrations in sausages such as dehydrated sausages, raw ham or cooked meats (DABIN; JUSSIAUX, 1994). This microorganism is capable of multiplying at high salt concentrations (up to 10% NaCl), although salt tolerance varies with pH and temperature conditions (AFSSA, 2000). *L. monocytogenes* strains that have adapted to the acid environment may become resistant to several harmful factors, including osmotic stress (O'DRISCOLL; GAHAN; HILL, 1996). However, adaptation to osmotic stress does not induce strong cross-protection against other stressors (LOU; YOUSEF, 1997).

Water activity is strongly linked to pH and salt concentrations of the content of meat products. For example, during the drying stage of cured raw sausage products (eg, sausage and sausage), the water holding capacity decreases, as the pH approaches the isoelectric point (TYÖPPÖNEN; PETÄJÄ; MATTILA-SANDHOLM, 2003). The addition of salt to the inlay mixture limits water activity, thereby inhibiting the growth of many pathogenic and spoilage bacteria including *L. monocytogenes*. After drying, the water activity is < 0.90, which also inhibits bacterial growth. Unfortunately, some food producers tend to reduce drying times to increase profitability. Products with insufficient drying may contain levels of water activity that allow the growth of *L. monocytogenes* (Aw = 0.92) (AFSSA, 2000), and are associated with a higher risk of listeriosis.

L. monocytogenes may be subjected to other potential stress in meat products. Microbial growth in certain meat products can consume all the oxygen in the matrix. The multiplication of aerobic deterioration bacteria will be affected, but not that of *L. monocytogenes* because it is an aerobic/anaerobic microorganism. Encinas *et al.*, (1999) found significant differences in the number of chouriço strains formulated with hot and soft paprika types. Other spices, such as pepper, cardamom and garlic, may also have antioxidant and antimicrobial properties (TYÖPPÖNEN; PETÄJÄ; MATTILA-SANDHOLM, 2003).

Finally, smoking, which contains phenols, carbonates and different organic acids, can inhibit different bacteria on the surfaces of cured pork meat. According to Encinas *et al.*, (1999), the smoking process in the manufacture of sausages and ham significantly reduces the contamination by *L. monocytogenes*.

4 Cleaning, disinfecting products and procedures on food processing lines

Typical processes of cleaning and disinfection in food processing environments involve a series of steps, each with a specific purpose. A pre-rinse is carried out using water to remove the coarse, loose material, and then cleaning with suitable detergents (usually alkaline) in order to remove residual substances. The detergent is then rinsed, the chemical antimicrobials are applied to sanitize surfaces and a final post-rinse with water removes these chemicals. The cleaning step is important because it removes the organic matter from processing surfaces (CHASSEIGNAUX *et al.*, 2001; THÉVENOT *et al.*, 2005c).

In addition to harboring bacteria, organic matter may reduce the efficiency of disinfectants (such as quaternary ammonium compounds - QACs) or prevent disinfectants from reaching bacteria at recommended concentrations (GIBSON *et al.*, 1999). Taormina *et al.*, (2002) showed that strains of *L. monocytogenes* that were exposed to cleaning solutions that did not affect cell viability were more sensitive to treatment with sanitizing chemicals.

Biofilms formed with multiple bacterial species, which combine the effects of shielding and production of extracellular polymers, also increase bacterial resistance to antimicrobial agents (COSTERTON *et al.*, 1995). Finally, food and food processing line equipment, along with the typical characteristics of the meat production system, offer microorganisms a complex environment that suppresses the effectiveness of antimicrobial agents (KORBER *et al.*, 2002).

Numerous studies have focused on sanitizing products (disinfection) and their effects *on L. monocytogenes*. In a study by Holah *et al.*, (2002), QAC's were markedly the most commonly used disinfectant in the food industry. This is mainly due to its biocide activity which causes widespread damage to the membrane and inactive cellular enzymes, combined with its non-toxic, non-corrosive nature to the skin and hard surfaces, which makes them ideal as a choice in manual application in the food industry.Mereghetti *et al.*, (2000) described a minimal inhibitory concentration of QACs that increased the resistance of *L. monocytogenes* strains isolated from a variety of environments, foods and animals.

Finally, Holah *et al.*, (2002) suggested that *L. monocytogenes* persistence does not occur due to the resistance to disinfectants, but to the physical resistance to the mechanisms of adaptation (superficial fixation, biofilm formation, reduced multiplication rate and quiescence). Several studies have emphasized the importance of using appropriate procedures and concentrations during the hygienic processes. For example, concentrations of sodium hypochlorite recommended by suppliers are more than sufficient to eliminate floating *L. monocytogenes*, but are ineffective in bacteria in multispecies biofilms grown on a stainless steel surface (NORWOOD; GILMOUR, 2000).

There is also a significant difference in the efficacy of sanitizers against *L. monocytogenes* when applied to stainless steel or conveyor belt surfaces (BREMER; MONK; BUTLER, 2002). This study suggested that the decreased efficacy of chlorine on the surface of the conveyor belt was due to the limited penetration of the disinfectant: the bacteria were relatively protected by the tissue. The authors concluded that during the hygiene process, little consideration is given to the physical properties of the material to be sanitized.

Authors also tested different procedures to improve sanitation processes. For example, Vasseur *et al.*, (2001) tested combinations of chemicals that could decrease or eliminate surviving *L. monocytogenes* from food processing plants. Chemicals that induce pH shocks were more efficient when used in combination with other parameters. An acid treatment followed by an alkaline treatment was not very effective against *L. monocytogenes*, while the opposite combination led to a 3 log reduction of the bacterial population. Whatever the bacterial species, the most efficient treatments were combinations of acids and alkaline, osmotic and biocidal shocks.

The equipment used in food processing plays an important role in maintaining contamination of the environment and contamination of processed foodstuffs (AUTIO, TIINA *et al.*, 2002; REIJ; DEN AANTREKKER; FORCE, 2004). In addition, it is difficult to eradicate *L. monocytogenes* from contaminated processing lines and equipment (AUTIO, TIINA *et al.*, 2003; MIETTINEN; BJÖRKROTH; KORKEALA, 1999).

Mechanical cleaning is the most effective way to separate adhered cells and biofilms. However, poorly designed equipment will have small spaces and narrow openings that are difficult or impossible to achieve, resulting in poor mechanical cleaning. These sites are the main danger of cross-contamination of pathogens that inhabit a mature biofilm (LUNDÉN *et al.*, 2003).

Sanitizers may not reach the contamination site in effective concentrations when applied according to the manufacturer's recommendations. It may be necessary to use disinfectant in higher concentrations in these specific areas of the food processing environment. Attention should be given to a correct design of the equipment and, in addition, useful recommendations and guidelines for the validation of the equipment's cleaning capacity should be published (REIJ; DEN AANTREKKER; FORCE, 2004).

Finally, compartmentalization of the processing line and disassembly of processing machines should help in the control of *L. monocytogenes* contamination in meat processing plants (AUTIO *et al.*, 2003; LUNDÉN *et al.*, 2003). In summary, the cleaning and disinfecting procedures of food processing machines combine mechanical energy and chemicals. When these procedures are properly applied, strains of *L. monocytogenes* persistent or not can be eliminated (REIJ; DEN AANTREKKER; FORCE, 2004).

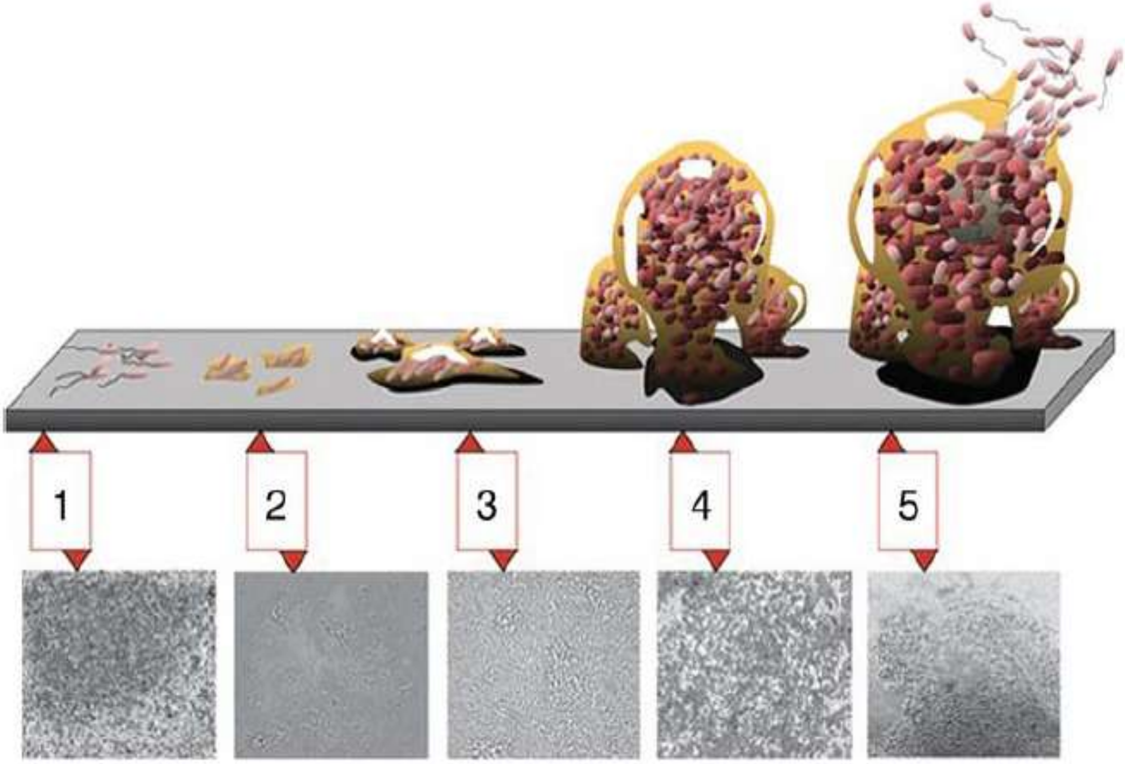
5 Biofilms in the food processing industry

Biofilms are multicellular microbial communities bound to surfaces or associated with interfaces and incorporated into self-produced or even acquired hydrated extracellular polymer (EPS) matrices (DAVEY; O'TOOLE, 2000; STOODLEY *et al.*, 2002). This is considered a strategy to support the persistence of microorganisms in most environments, both natural and artificial (HALL-STOODLEY; STOODLEY, 2005). Biofilm development involves the initial adhesion of planktonic (free) cells to a surface, followed by replication and production of extracellular polymeric substances (EPS), formation of microcolonies, maturation (development of the biofilm architecture) and detachment (GIAOURIS, EFSTATHIOS E.; SIMÕES, 2018) (Figure 1).

Microorganisms change from the planktonic state to biofilm through a complex and highly regulated mechanism that is influenced by environmental conditions (eg, low nutrient availability or high population density). Within a biofilm population, cells with diverse genotypes and phenotypes, expressing distinct metabolic pathways, stress responses and other specific biological activities, may be closely correlated (STEWART; FRANKLIN, 2008).

Interestingly, many bacteria, including foodborne pathogens, are known to control their metabolism through a process called quorum sensing (QS), in which cells communicate by synthesizing, detecting, and responding to small, diffusible signaling molecules called auto-inducers (IA) or bacterial pheromones (SKANDAMIS; NYCHAS, 2012). Since biofilms normally contain a high concentration of cells, AI activity, QS cell density dependence, and regulation of gene expression have been proposed as essential components of biofilm physiology (AN; PARSEK, 2007).

Figure 1. Biofilm Life Cycle Steps: 1, initial adhesion; 2, replication and EPS production; 3, formation of microcolonies; 4, maturation (development of the biofilm architecture); 5, detachment. Each stage of the life cycle in the diagram is paired with a photomicrograph of a developing biofilm of *Pseudomonas aeruginosa*.



Biofilm formation is believed to offer significant ecological advantages to microorganisms, and more particularly: (1) protection against conditions such as antimicrobial and chemical agents, nutritional and oxidative stress, heat and acid challenges, exposure to UV light, changes in pH, osmotic shock and desiccation (BRIDIER *et al.*, 2015), (2) availability of nutrients and cooperativity of metabolites (DAVEY; O'TOOLE, 2000), and (3) acquisition of new traits with communities of biofilms, for horizontal gene transfer (MADSEN *et al.*, 2012).

Biofilms formed on food contact surfaces are of considerable interest in the context of food hygiene, since they may contain both deteriorating and pathogenic bacteria, which may lead to contamination of the post-processing environment, leading to products with short shelf life and pathogens transmission. In addition to the significant incidence of foodborne diseases in public health, there are also huge economic costs, which are estimated to exceed $ 50 billion annually in the United States alone (SCHARFF, 2012).

From an ecological point of view, food processing facilities could be considered as microbial habitats constantly disrupted by hygienic procedures (VALDERRAMA; CUTTER, 2013), most foodborne bacteria are able to bind to various surfaces found in these areas and form biofilms, where they can persist and survive for long periods depending on the stress and surrounding environmental conditions (WINKELSTRÖTER *et al.*, 2014). Thus, biofilms formed in the industrial environment are difficult to remove, often resulting in persistent strains and endemic populations (ABDALLAH *et al.*, 2014). Persistent strains of *L. monocytogenes* were found to exhibit higher adhesion and shorter contact time to stainless steel (SS) surfaces when compared to non-persistent strains, promoting their survival in food processing facilities and possibly having an effect at the beginning of the persistent contamination of the plants (LUNDEN *et al.*, 2000).

In recent decades, the formation of biofilms in the food industry by bacterial pathogens such as *Salmonella* spp., *L. monocytogenes*, pathogenic *Escherichia coli*, *Campylobacter* spp., *Bacillus cereus* and *Staphylococcus aureus*, has attracted a lot of attention, since these microorganisms within biofilms are protected from sanitizers, increasing the likelihood of survival and subsequent food contamination (CHMIELEWSKI; FRANK, 2003).

An unique feature of biofilms is that, since they have been developed in food processing facilities and equipment, they are difficult to eradicate, mainly because of their stability and extremely strong matrix (GIAOURIS; SIMÕES, 2018). It covers cells and contains EPS, such as bacterial exopolysaccharides, sugars, proteins, lipids, teichoic acids, nucleic acids, and other minor components. All of these provide the biofilm with mechanical stability, and measure its strong adhesion to the surfaces and form a three-dimensional network of cohesive polymer that interconnects and transiently immobilizes the involved cells (FLEMMING; WINGENDER, 2010).

Generally, increased resistance of surface-adhered microbial communities to antimicrobial agents may be, in parallel, due to: 1) limitations to the free diffusion of antimicrobial agents through the biofilm matrix; 2) variability in the physical and chemical microenvironments within the biofilm (for example, varied conditions of pH, osmotic force or nutrients), leading to varying levels of metabolic activity of the biofilm cells and also to the modification of the sanitizing efficiency; 3) adaptive responses to stress, resulting from mutations, altered gene expression and also through possible horizontal transfer of resistance coding genes; 4) differentiation of bacterial cells in physiological states less susceptible to treatments (eg, latent, viable but not cultivable - VBNC, persistent) (GIAOURIS; NESSE, 2015). There is also growing evidence that interspecies interactions may deeply increase biofilm resistance to biocides, in particular, the protection of disinfectant treatments of pathogenic species by resident surface microbiota.

Modern food processing provides an environment for the formation of biofilm on surfaces, for example, the great complexity of processing equipment (hampering proper hygiene), mass production of products, long production cycles and the wide surface areas available for biofilm development (LINDSAY; VON HOLY, 2006).

Conveyor belts, food boxes and cutting tools are highly subject to contamination due to frequent use and are important contributors to cross-contamination through direct contact with food products (GIAOURIS *et al*., 2014). Such surfaces are worn away with repeated use and control of microbial contamination in such materials in contact with food often requires differentiated cleaning and disinfection approaches. Generally, food processing plants that employ effective daily sanitation programs on equipment should not have true biofilms on most food contact surfaces. However, these can be formed on surfaces that may not receive sufficient exposure to chemical sanitizing products such as drains, walls, ceilings, pipelines, pumps, valves, gaskets and corners (also called "dead zones"). Cracks and crevices can also provide hiding places for microorganisms (GIAOURIS; SIMÕES, 2018).

All these surfaces can be reservoir of pathogenic bacteria forming complex communities of multispecies biofilms with another environmental microbiota, which may help them avoid being removed or inactivated by disinfectants (LIU *et al*., 2016). As an example, *L. monocytogenes* can survive in *Pseudomonas* biofilms (PUGA; ORGAZ; SANJOSE, 2016). The sequencing of 16S rRNA genes from bacterial communities of drained water and drainage biofilms in a food processing environment contaminated with *L. monocytogenes* revealed 16 clones dominated by *Proteobacteria*, *Firmicutes* and *Bacteroidetes* (DZIECIOL *et al*., 2016). Under such conditions, cell-to-cell interactions are inevitable and may lead to the establishment of dense, complex and highly structured populations of biofilms capable of coordinated and collective behavior (GIAOURIS; SIMÕES, 2018).

In any food processing environment, food residues adsorbed onto a substrate create film conditioning that may affect, improving or reducing bacterial adhesion. In addition, bacterial survival and growth may be motivated by the presence of food residues that may increase the resistance of bacteria adhering to desiccation, for example, also rendering sanitizing processes ineffective and encouraging cross-contamination (KUDA *et al.*, 2015).

Most chemical cleaning agents used in the food processing industry are alkaline compounds or acids that act as detergents for fat/protein and precipitated minerals, respectively (CHMIELEWSKI; FRANK, 2003). These substances suspend and dissolve food residues by reducing surface tension, emulsifying fats, denaturing proteins and can be used in combination with chelating agents and anionic wetting agents (compatible with acid or alkaline cleaners) (SIMÕES; SIMOES; VIEIRA, 2010).

An effective cleaning procedure should effectively remove food debris and other solids that may contain microorganisms or promote microbial multiplication and also break or dissolve the biofilm-associated EPS matrix so that sanitizing agents can gain access to viable cells (GIBSON *et al.*, 1999). However, surfaces that look visually clean can still be contaminated with a large number of viable microorganisms that could contaminate food. Therefore, following the removal of food waste, additional disinfection measures are required to reduce the number of microorganisms present. Disinfection is especially important in food handling environments (GIAOURIS; SIMÕES, 2018).

If sanitizing procedures are not followed correctly, biofilm-producing bacteria can survive and progressively adapt to the environment, which makes them even more difficult to remove through sanitization methods (CHAITIEMWONG; HAZELEGER; BEUMER, 2010). In addition, conventional cleaning and disinfection strategies employed in food production facilities, including chemical agents and physical treatments, may not deal proficiently with biofilm-related problems and may also contribute to the spread of resistance (YANG, LIANG *et al.*, 2012).

Standards for testing the activity of bactericide of chemical disinfectants such as the European quantitative suspension tests EN 1040 and 1276 (European Standard), use widely planktonic cultures and the results do not necessarily reflect the efficacy against biofilm bacteria (GIAOURIS; SIMÕES, 2018). It has been suggested that the action of some biocides may strengthen the attachment of bacteria to a surface rather than remove or weaken its adhesion.

These questions led to a growing interest in the use of additional resources or alternative disinfectants, eg, enzymes, bacteriophages, ultrasound, electrolyzed oxidizing water and ozone (MEIRELES *et al.*, 2016). There is an urgent need to develop new strategies to control robust biofilms in industrial and clinical settings. In recent years, a wide range of promising approaches have been successfully evaluated in different biofilm model systems (GIAOURIS; SIMÕES, 2018). Currently, there is interest in using organic biocides and environmentally friendly alternatives in food processing environments because of the potential dangers of chemical and synthetic agents to public health and the environment (ASHRAF *et al.*, 2014).

Bibliography

ABDALLAH, Marwan *et al.* Biofilm formation and persistence on abiotic surfaces in the context of food and medical environments. *Archives of microbiology*, v. 196, n. 7, p. 453–472, 2014.

ALIŞARLI, M; ATASEVER, M; GÖKMEN, M. Contamination of some vacuum-packaged meat productswith *Listeria monocytogenes*. Acta Alimentaria, v. 34, n. 3, p. 331–334, 2005.

AN, D.; PARSEK, M. R. The promise and peril of transcriptional profiling in biofilm communities. Current opinion in Microbiology, v. 10, n. 3, p. 292–296, 2007.

ANDRITSOS, N. D. *et al.* Quantifying *Listeria monocytogenes* prevalence and concentration in minced pork meat and estimating performance of three culture media from presence/absence microbiological testing using a deterministic and stochastic approach. Food Microbiology, v. 36, n. 2, p. 395–405, 2013.

ANGELIDIS, A. S; KOUTSOUMANIS, K. Prevalence and concentration of *Listeria monocytogenes* in sliced ready-to-eat meat products in the Hellenic retail market. Journal of Food Protection, v. 69, n. 4, p. 938–942, 2006.

ASHRAF, M. A. *et al.* Green biocides, a promising technology: current and future applications to industry and industrial processes. Journal of the Science of Food and Agriculture, v. 94, n. 3, p. 388–403, 2014.

AUTIO, T. *et al. Listeria monocytogenes* contamination pattern in pig slaughterhouses. Journal of Food Protection, v. 63, n. 10, p. 1438–1442, 2000.

AUTIO, T. *et al.* Characterisation of persistent and sporadic *Listeria monocytogenes* strains by pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). Systematic and Applied Microbiology, v. 26, n. 4, p. 539–545, 2003.

AUTIO, T. *et al.* Similar *Listeria monocytogenes* pulsotypes detected in several foods originating from different sources. International Journal of Food Microbiology, v. 77, n. 1–2, p. 83–90, 2002.

AZIZOGLU, R. O. *et al.* Resistance of *Listeria monocytogenes* biofilms to sanitizing agents. Biofilms in the Food Environment, p. 51, 2015.

BARROS, M. A. F. *et al. Listeria monocytogenes*: Occurrence in beef and identification of the main contamination points in processing plants. Meat Science, v. 76, n. 4, p. 591–596, 2007.

BERSOT, L. dos S. *et al.* Behaviour of *L. monocytogenes* in sliced, vacuum-packed mortadella. Brazilian Journal of Microbiology, v. 39, n. 3, p. 514–516, 2008.

BERZINS, A. *et al.* Factors associated with *Listeria monocytogenes* contamination of cold-smoked pork products produced in Latvia and Lithuania. International Journal of Food Microbiology, v. 115, n. 2, p. 173–179, 2007.

BĒRZIŅS, A.; TERENTJEVA, M.; KORKEALA, H. Prevalence and genetic diversity of *Listeria monocytogenes* in vacuum-packaged ready-to-eat meat products at retail markets in Latvia. Journal of Food Protection, v. 72, n. 6, p. 1283–1287, 2009.

BORUCKI, M. K.; CALL, D. R. *Listeria monocytogenes* serotype identification by PCR. Journal of Clinical Microbiology, v. 41, n. 12, p. 5537–5540, 2003.

BOTTELDOORN, N. *et al. Salmonella* on pig carcasses: positive pigs and cross contamination in the slaughterhouse. Journal of Applied Microbiology, v. 95, n. 5, p. 891–903, 2003.

BREMER, P. J.; MONK, I.; BUTLER, R. Inactivation of *Listeria monocytogenes* / *Flavobacterium* spp. biofilms using chlorine: impact of substrate, pH, time and concentration. Letters in applied microbiology, v. 35, n. 4, p. 321–325, 2002.

BRIDIER, A. *et al.* Biofilm-associated persistence of food-borne pathogens. Food microbiology, v. 45, p. 167–178, 2015.

BRIDIER, A. *et al.* Resistance of bacterial biofilms to disinfectants: a review. Biofouling, v. 27, n. 9, p. 1017–1032, 2011.

BUCHANAN, R. L.; PHILLIPS, J. G. Response surface model for predicting the effects of temperature pH, sodium chloride content, sodium nitrite concentration and atmosphere on the growth of *Listeria monocytogenes*. Journal of Food Protection, v. 53, n. 5, p. 370–376, 1990.

CABANES, D. *et al.* Surface proteins and the pathogenic potential of *Listeria monocytogenes*. Trends in Microbiology, v. 10, n. 5, p. 238–245, 2002.

CABEÇA, T. K. Suscetibilidade de microorganismos relacionados com a contaminação de alimentos em biofilme artificial e em suspensão frente a desinfetantes. 2006.

CABEÇA, T. K.; PIZZOLITTO, A. C.; PIZZOLITTO, E. L. Activity of disinfectants against foodborne pathogens in suspension and adhered to stainless steel surfaces. Brazilian Journal of Microbiology, v. 43, n. 3, p. 1112–1119, 2012.

CALY, D. *et al.* Sodium chloride affects *Listeria monocytogenes* adhesion to polystyrene and stainless steel by regulating flagella expression. Letters in applied microbiology, v. 49, n. 6, p. 751–756, 2009.

CAMARGO, A. C. *et al. Listeria monocytogenes* in Food-Processing Facilities, Food Contamination, and Human Listeriosis: The Brazilian Scenario. Foodborne Pathogens and Disease, v. 14, n. 11, p. fpd.2016.2274, 2017. Disponível em: <http://online.liebertpub.com/doi/10.1089/fpd.2016.2274>.

CAMARGO, A. C. *et al.* Low occurrence of *Listeria monocytogenes* on bovine hides and carcasses in Minas Gerais State, Brazil: molecular characterization and antimicrobial resistance. Journal of Food Protection, v. 77, n. 7, p. 1148–1152, 2014.

CAMPDEPADRÓS, M. *et al.* Effectiveness of two sanitation procedures for decreasing the microbial contamination levels (including *Listeria monocytogenes*) on food contact and non-food contact surfaces in a dessert-processing factory. Food Control, v. 23, n. 1, p. 26–31, 2012.

CARANDINA, D. C. F. Avaliação de biofilmes formados por isolados de *Listeria monocytogenes* provenientes de laticínios e perfil de resistência a agentes sanitizantes.[S.l.]: Universidade de São Paulo. , 2013

CHAE, M. S.; SCHRAFT, H. Comparative evaluation of adhesion and biofilm formation of different *Listeria monocytogenes* strains. International Journal of Food Microbiology, v. 62, n. 1–2, p. 103–111, 2000.

CHAITIEMWONG, N.; HAZELEGER, W. C.; BEUMER, R. R. Survival of *Listeria monocytogenes* on a conveyor belt material with or without antimicrobial additives. International Journal of Food Microbiology, v. 142, n. 1–2, p. 260–263, 2010.

CHANG, Y. *et al.* Identification of genes involved in *Listeria monocytogenes* biofilm formation by mariner-based transposon mutagenesis. Applied Microbiology and Biotechnology, v. 93, n. 5, p. 2051–2062, 2012.

CHARLIER, C. *et al.* Clinical features and prognostic factors of listeriosis: the MONALISA national prospective cohort study. The Lancet Infectious Diseases, v. 17, n. 5, p. 510–519, 2017.

CHASSEIGNAUX, E. *et al.* Molecular epidemiology of *Listeria monocytogenes* isolates collected from the environment, raw meat and raw products in two poultry and pork-processing plants. Journal of Applied Microbiology, v. 91, n. 5, p. 888–899, 2001.

CHMIELEWSKI, R. A. N.; FRANK, J. F. Biofilm formation and control in food processing facilities. Comprehensive Reviews in Food Science and Food Safety, v. 2, n. 1, p. 22–32, 2003.

CLSI. M45. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria ; Proposed Guideline. Guidelines CLSI, v. 35, n. 17, p. 24, 2015.

CLSI, CaLSI. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard—third edition—document m27-a3. CLSI, Wayne, Pa, USA, 2008.

COLAK, H. *et al.* Presence of *Listeria monocytogenes* in Turkish style fermented sausage (sucuk). Food Control, v. 18, n. 1, p. 30–32, 2007.

CONTER, M. *et al.* Characterization of antimicrobial resistance of foodborne *Listeria monocytogenes*. International Journal of Food Microbiology, v. 128, n. 3, p. 497–500, 2009.

COTTER, P. D. *et al.* Identification and disruption of lisRK, a genetic locus encoding a two-component signal transduction system involved in stress tolerance and virulence in *Listeria monocytogenes*. Journal of bacteriology, v. 181, n. 21, p. 6840–6843, 1999.

COTTER, P. D.; HILL, C. Surviving the acid test: responses of gram-positive bacteria to low pH. Microbiology and Molecular Biology Reviews, v. 67, n. 3, p. 429–453, 2003.

CURVELO, P. *et al.* Occorrencia de *Listeria monocytogenes* em produtos de carne de Peru comercializados na cidade de Niteroi-RJ-Brasil. Acta Scientiae Veterinae, v. 30, p. 19–25, 2002.

DA SILVA, D. A. L. *et al.* Hygiene and Safety in the Meat Processing Environment from Butcher Shops: Microbiological Contamination and *Listeria monocytogenes*. Journal of Food Protection, v. 79, n. 4, p. 628–634, 2016.

DABIN, E.; JUSSIAUX, R. Modifications des saucissons au cours de l'étuvage et du séchage. Le saucisson sec. Erti, Paris, p. 136–138, 1994.

DAVEY, M. E.; O'TOOLE, G. A. Microbial biofilms: from ecology to molecular genetics. Microbiology and Molecular Biology Reviews, v. 64, n. 4, p. 847–867, 2000.

DE CESARE, A.; MIONI, R.; MANFREDA, G. Prevalence of *Listeria monocytogenes* in fresh and fermented Italian sausages and ribotyping of contaminating strains. International Journal of Food Microbiology, v. 120, n. 1–2, p. 124–130, 2007.

DE VALK, H. *et al.* Two consecutive nationwide outbreaks of listeriosis in France, October 1999–February 2000. American Journal of Epidemiology, v. 154, n. 10, p. 944–950, 2001.

DEGENHARDT, R.; SANT'ANNA, E. S. Pesquisa de *Listeria* sp. em embutidos cárneos fermentados produzidos na região Meio-Oeste de Santa Catarina, Brasil. Boletim do CEPPA, v. 25, n. 1, p. 133–140, 2007.

DI BONAVENTURA, G. *et al.* Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food contact surfaces: relationship with motility and cell surface hydrophobicity. Journal of Applied Microbiology, v. 104, n. 6, p. 1552–1561, 2008.

DJORDJEVIC, D.; WIEDMANN, M.; MCLANDSBOROUGH, L. A. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Applied and Environmental Microbiology, v. 68, n. 6, p. 2950–2958, 2002.

DOUMITH, M. *et al.* Differentiation of the Major *Listeria monocytogenes* Serovars by Multiplex PCR Differentiation of the Major *Listeria monocytogenes* Serovars by Multiplex PCR. Journal of Clinical Microbiology, v. 42, n. 8, p. 3819–3822, 2004.

DUFFY, E. A. *et al.* Extent of microbial contamination in United States pork retail products. Journal of Food Protection, v. 64, n. 2, p. 172–178, 2001.

DZIECIOL, M. *et al.* Bacterial diversity of floor drain biofilms and drain waters in a *Listeria monocytogenes* contaminated food processing environment. International Journal of Food Microbiology, v. 223, p. 33–40, 2016.

ENCINAS, J. P. *et al.* Behaviour of *Listeria* spp. in naturally contaminated chorizo (Spanish fermented sausage). International Journal of Food Microbiology, v. 46, n. 2, p. 167–171, 1999.

FAI, A. E. C. *et al. Salmonella* sp e *Listeria monocytogenes* em presunto suíno comercializado em supermercados de Fortaleza (CE, Brasil): fator de risco para a saúde pública. Ciência & Saúde Coletiva, v. 16, p. 657–662, 2011.

FALEIRO, M. L.; ANDREW, P. ; POWER, D. Stress response of *Listeria monocytogenes* isolated from cheese and other foods. International Journal of Food Microbiology, v. 84, n. 2, p. 207–216, 2003.

FARBER, J. M.; PETERKIN, P. I. *Listeria monocytogenes*, a food-borne pathogen. *Microbiological reviews*, v. 55, n. 3, p. 476–511, 1991.

FÉLIX, B. *et al.* Population Genetic Structure of *Listeria monocytogenes* Strains Isolated From the Pig and Pork Production Chain in France. Frontiers in Microbiology, v. 9, p. 684, 2018.

FENLON, D. R.; WILSON, J.; DONACHIE, W. The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing. *Journal of Applied Microbiology*, v. 81, n. 6, p. 641–650, 1996.

FENLON, D. R. *Listeria monocytogenes* in the natural environment. Food Science and Technology-New York-Marcel Dekker-, p. 21–38, 1999.

FERREIRA, V. *et al. Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. Journal of Food Protection, v. 77, n. 1, p. 150–170, 2014.

FLEMING, D. W. *et al.* Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. New England Journal of Medicine, v. 312, n. 7, p. 404–407, 1985.

FLEMMING, H. C.; WINGENDER, J. The biofilm matrix. Nature Reviews Microbiology, v. 8, n. 9, p. 623, 2010.

FOSSE, J. ; SEEGERS, H. ; MAGRAS, C. Prevalence and Risk Factors for Bacterial Food-Borne Zoonotic Hazards in Slaughter Pigs: A Review. Zoonoses and Public Health, v. 56, n. 8, p. 429–454, 2009.

GALVÃO, N. N. *et al.* PFGE characterisation and adhesion ability of *Listeria monocytogenes* isolates obtained from bovine carcasses and beef processing facilities. Meat Science, v. 92, n. 4, p. 635–643, 2012.

GANDRA, T. K. V. *et al.* Expression levels of the agr locus and prfA gene during biofilm formation by *Listeria monocytogenes* on stainless steel and polystyrene during 8 to 48 h of incubation 10 to 37° C. International journal of Food Microbiology, v. 300, p. 1–7, 2019.

GIANFRANCESCHI, M. *et al.* Survival of *Listeria monocytogenes* in uncooked Italian dry sausage (salami). Journal of Food Protection, v. 69, n. 7, p. 1533–1538, 2006.

GIAOURIS, E. *et al.* Attachment and biofilm formation by foodborne bacteria in meat processing environments: Causes, implications, role of bacterial interactions and control by alternative novel methods. Meat Science, v. 97, n. 3, p. 289–309, 2014.

GIAOURIS, E. E.; S., M. V. Pathogenic Biofilm Formation in the Food Industry and Alternative Control Strategies. [S.l.]: Elsevier Inc., 2018. v. 15. Disponível em: <http://dx.doi.org/10.1016/B978-0-12-811444-5/00011-7>.

GIAOURIS, E.; NESSE, L. L. Attachment of *Salmonella* spp. to food contact and product surfaces and biofilm formation on them as stress adaptation and survival strategies. *Salmonella*: Prevalence, Risk Factors and Treatment Options, p. 111–136, 2015.

GIBSON, H. *et al.* Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. Journal of Applied Microbiology, v. 87, n. 1, p. 41–48, 1999.

GIL, M. I. *et al.* Fresh-cut product sanitation and wash water disinfection: problems and solutions. International Journal of Food Microbiology, v. 134, n. 1–2, p. 37–45, 2009.

GILLESPIE, I.; LITTLE, C. and; MITCHELL, R. Microbiological examination of cold ready-to-eat sliced meats from catering establishments in the United Kingdom. Journal of Applied Microbiology, v. 88, n. 3, p. 467–474, 2000.

GIOVANNACCI, I. *et al. Listeria monocytogenes* in pork slaughtering and cutting plants: use of RAPD, PFGE and PCR–REA for tracing and molecular epidemiology. International Journal of Food Microbiology, v. 53, n. 2–3, p. 127–140, 1999.

GOULET, V. *et al. Listeriosis* outbreak associated with the consumption of rillettes in France in 1993. Journal of Infectious Diseases, v. 177, n. 1, p. 155–160, 1998.

GRAVES, L. M.; SWAMINATHAN, B. PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. International journal of food microbiology, v. 65, n. 1, p. 55–62, 2001.

GREENWOOD, M. H; R., D.; BURDEN, P. The occurrence of *Listeria* species in milk and dairy products: a national survey in England and Wales. International Journal of Food Microbiology, v. 12, n. 2–3, p. 197–206, 1991.

GUERIRI, I. *et al.* The DegU orphan response regulator of *Listeria monocytogenes* autorepresses its own synthesis and is required for bacterial motility, virulence and biofilm formation. Microbiology, v. 154, n. 8, p. 2251–2264, 2008.

GUESCINI, M. *et al.* A new real-time PCR method to overcome significant quantitative inaccuracy due to slight amplification inhibition. BMC Bioinformatics, v. 9, n. 1, p. 326, 2008.

HALL-STOODLEY, L.; STOODLEY, P. Biofilm formation and dispersal and the transmission of human pathogens. Trends in Microbiology, v. 13, n. 1, p. 7–10, 2005.

HARTER, E. *et al.* Stress survival islet 2, predominantly present in *Listeria monocytogenes* strains of sequence type 121, is involved in the alkaline and oxidative stress responses. *Appl.* Environmental Microbiology, v. 83, n. 16, p. e00827-17, 2017.

HELLSTRÖM, S. *et al. Listeria monocytogenes* Contamination in Pork Can Originate from Farms. Journal of Food Protection, v. 73, n. 4, p. 641–648, 2010. Disponível em: <http://jfoodprotection.org/doi/abs/10.4315/0362-028X-73.4.641>.

HERIGSTAD, B.; HAMILTON, M.; HEERSINK, J. How to optimize the drop plate method for enumerating bacteria. *Journal of microbiological methods*, v. 44, n. 2, p. 121–129, 2001.

HOF, H.; NICHTERLEIN, T.; KRETSCHMAR, M. When are *Listeria* in foods a health risk? Trends in Food Science & Technology, v. 5, n. 6, p. 185–190, 1994.

HOF, H.; ROCOURT, J. Is any strain of *Listeria monocytogenes* detected in food a health risk? International Journal of Food Microbiology, v. 16, n. 3, p. 173–182, 1992.

HOLAH, J. T. *et al.* Biocide use in the food industry and the disinfectant resistance of persistent strains of *Listeria monocytogenes* and Escherichia coli. Journal of applied microbiology, v. 92, n. s1, 2002.

HOOD, S. K.; ZOTTOLA, E. A. Biofilms in food processing. *Food control*, v. 6, n. 1, p. 9–18, 1995.

HUDSON, J. A. *et al.* Rapid detection of *Listeria monocytogenes* in ham samples using immunomagnetic separation followed by polymerase chain reaction. Journal of Applied Microbiology, v. 90, n. 4, p. 614–621, 2001.

ICHIRO SAKATE, Ricardo *et al.* Quantificação de *Listeria monocytogenes* em salames fatiados embalados a vácuo. Archivos Latinoamericanos de Nutricion, v. 53, n. 2, p. 184–187, 2003.

INOUE, S. *et al.* Prevalence and contamination levels of *Listeria monocytogenes* in retail foods in Japan. International Journal of Food Microbiology, v. 59, n. 1–2, p. 73–77, 2000.

ISO, E N. 11290-1: Microbiology of food and animal feeding stuffs-horizontal method for the detection and enumeration of *Listeria monocytogenes*-Part 1: detection method. Geneva: International Organization for Standardization, 1996.

ISO, I S O. 11290-1/Amd 1—Modification of the Isolation Media and the Haemolysis Test, and Inclusion of Precision Data. Geneva*:* ISO, 2004.

JANAKIRAMAN, Vanitha. *Listeriosis* in pregnancy: diagnosis, treatment, and prevention. Reviews in Obstetrics and Gynecology, v. 1, n. 4, p. 179, 2008.

JAY, J. M. Prevalence of *Listeria* spp. in meat and poultry products. Food Control, v. 7, n. 4–5, p. 209–214, 1996.

JEFFERSON, K. K. What drives bacteria to produce a biofilm? FEMS Microbiology Letters, v. 236, n. 2, p. 163–173, 2004.

JEMMI, T.; PAK, S. I.; SALMAN, M. D. Prevalence and risk factors for contamination with *Listeria monocytogenes* of imported and exported meat and fish products in Switzerland, 1992–2000. Preventive Veterinary Medicine, v. 54, n. 1, p. 25–36, 2002.

JENSEN, A. *et al.* Processing plant persistent strains of *Listeria monocytogenes* appear to have a lower virulence potential than clinical strains in selected virulence models. International Journal of Food Microbiology, v. 123, n. 3, p. 254–261, 2008.

JENSEN, A. *et al.* Sodium chloride enhances adherence and aggregation and strain variation influences invasiveness of *Listeria monocytogenes* strains. Journal of Food Protection, v. 70, n. 3, p. 592–599, 2007.

JONES, E. M.; MACGOWAN, A. P. Antimicrobial chemotherapy of human infection due to *Listeria monocytogenes*. European Journal of Clinical Microbiology & Infectious Diseases, v. 14, n. 3, p. 165–175, 1995.

KALMOKOFF, M. L. *et al.* Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. Journal of Applied Microbiology, v. 91, n. 4, p. 725–734, 2001.

KANUGANTI, S. R. *et al.* Detection of *Listeria monocytogenes* in pigs and pork. Journal of Food Protection, v. 65, n. 9, p. 1470–4, 2002a.

KANUGANTI, S. R. *et al.* Detection of *Listeria monocytogenes* in pigs and pork. Journal of Food Protection, v. 65, n. 9, p. 1470–1474, 2002b.

KARAKOLEV, Rumen. Incidence of *Listeria monocytogenes* in beef, pork, raw-dried and raw-smoked sausages in Bulgaria. Food Control, v. 20, n. 10, p. 953–955, 2009.

KASTBJERG, Vicky Gaedt; GRAM, Lone. Model systems allowing quantification of sensitivity to disinfectants and comparison of disinfectant susceptibility of persistent and presumed nonpersistent *Listeria monocytogenes*. Journal of Applied Microbiology, v. 106, n. 5, p. 1667–1681, 2009.

KEENEY, K *et al.* Stress survival islet 1 contributes to serotype-specific differences in biofilm formation in *Listeria monocytogenes*. Letters in Applied Microbiology, v. 67, n. 6, p. 530–536, 2018.

KNUDSEN, G. M.; OLSEN, J. E.; DONS, L. Characterization of DegU, a response regulator in *Listeria monocytogenes*, involved in regulation of motility and contributes to virulence. FEMS Microbiology Letters, v. 240, n. 2, p. 171–179, 2004.

KORBER, D R *et al.* Efficacy enhancement of trisodium phosphate against spoilage and pathogenic bacteria in model biofilms and on adipose tissue. Journal of Food Protection, v. 65, n. 4, p. 627–635, 2002.

KOUTSOUMANIS, K. P.; KENDALL, P. A.; SOFOS, J. N. Effect of food processing-related stresses on acid tolerance of *Listeria monocytogenes*. Applied and Environmental Microbiology, v. 69, n. 12, p. 7514–7516, 2003.

KOVACEVIC, J. *et al.* Tolerance of *Listeria monocytogenes* to quaternary ammonium sanitizers is mediated by a novel efflux pump encoded by emrE. *Appl.* Environmental Microbiolog., v. 82, n. 3, p. 939–953, 2016.

KUDA, T. *et al.* Effect of quantity of food residues on resistance to desiccation of food-related pathogens adhered to a stainless steel surface. Food Microbiology, v. 46, p. 234–238, 2015.

LARIVIÈRE-GAUTHIER, G. *et al.* Analysis of *Listeria monocytogenes* strain distribution in a pork slaughter and cutting plant in the province of Quebec. Journal of Food Protection, v. 77, n. 12, p. 2121–2128, 2014.

LEBERT, I. *et al.* Diversity of microorganisms in the environment and dry fermented sausages of small traditional French processing units. Meat Science, v. 76, n. 1, p. 112–122, 2007.

LEMON, K. P.; HIGGINS, D. E.; KOLTER, R. Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. Journal of Bacteriology, v. 189, n. 12, p. 4418–4424, 2007.

LERICHE, VALÉRIE; CARPENTIER, BRIGITTE. Viable but nonculturable *Salmonella* typhimurium in single-and binary-species biofilms in response to chlorine treatment. Journal of Food Protection, v. 58, n. 11, p. 1186–1191, 1995.

LI, L. *et al.* Characterization of antimicrobial resistance of *Listeria monocytogenes* strains isolated from a pork processing plant and its respective meat markets in Southern China. Foodborne Pathogens and Disease, v. 13, n. 5, p. 262–268, 2016.

LINDSAY, D.; VON HOLY, A. What food safety professionals should know about bacterial biofilms. British Food Journal, v. 108, n. 1, p. 27–37, 2006.

LIU, D. Preparation of *Listeria monocytogenes* specimens for molecular detection and identification. International Journal of Food Microbiology, v. 122, n. 3, p. 229–242, 2008.

LIU, Yanlan *et al.* Molecular analysis of dominant species in *Listeria monocytogenes*-positive biofilms in the drains of food processing facilities. Applied Microbiology and Biotechnology, v. 100, n. 7, p. 3165–3175, 2016.

LOEPFE, C. *et al.* Reduced host cell invasiveness and oxidative stress tolerance in double and triple csp gene family deletion mutants of Listeria monocytogenes. Foodborne Pathogens and Disease, v. 7, n. 7, p. 775–783, 2010.

LOMONACO, S.; NUCERA, D.; FILIPELLO, V. The evolution and epidemiology of *Listeria monocytogenes* in Europe and the United States. Infection, Genetics and Evolution, v. 35, p. 172–183, 2015.

LÓPEZ, M. *et al.* Occurrence of contaminat biota in different European dry-sausages. Acta Alimentaria, v. 29, n. 3, p. 201–216, 2000.

LÓPEZ, V. *et al.* Molecular tracking of *Listeria monocytogenes* in an Iberian pig abattoir and processing plant. Meat Science, v. 78, n. 1–2, p. 130–134, 2008.

LOU, Y.; YOUSEF, A. E. Adaptation to sublethal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. *A*pplied and Environmental Microbiology, v. 63, n. 4, p. 1252–1255, 1997.

LUNDÉN, J. *et al.* Adaptive and cross-adaptive responses of persistent and non-persistent *Listeria monocytogenes* strains to disinfectants. International Journal of Food Microbiology, v. 82, n. 3, p. 265–272, 2003.

LUNDEN, J. M. *et al.* Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. Journal of Food Protection, v. 63, n. 9, p. 1204–1207, 2000.

MACHATA, S. *et al.* Simultaneous deficiency of both MurA and p60 proteins generates a rough phenotype in *Listeria monocytogenes*. Journal of Bacteriology, v. 187, n. 24, p. 8385–8394, 2005.

MACKEY, B. M. *et al.* Heat resistance of *Listeria*: strain differences and effects of meat type and curing salts. Letters in Applied Microbiology, v. 10, n. 6, p. 251–255, 1990.

MADSEN, Js S. *et al.* The interconnection between biofilm formation and horizontal gene transfer. FEMS Immunology & Medical Microbiology, v. 65, n. 2, p. 183–195, 2012.

MAFRA, I. *et al.* Comparative study of DNA extraction methods for soybean derived food products. Food Control, v. 19, n. 12, p. 1183–1190, 2008.

MARTÍN, B.; BOVER-CID, S.; AYMERICH, T. MLVA subtyping of *Listeria monocytogenes* isolates from meat products and meat processing plants. Food Research International, 2017.

MARTINEZ, I. *et al.* Genetic variability among isolates of *Listeria monocytogenes* from food products, clinical samples and processing environments, estimated by RAPD typing. International Journal of Food Microbiology, v. 84, n. 3, p. 285–297, 2003.

MARTINEZ, L.; REEVES, A.; HALDENWANG, W. Stressosomes formed in Bacillus subtilis from the RsbR protein of *Listeria monocytogenes* allow σB activation following exposure to either physical or nutritional stress. Journal of Bacteriology, v. 192, n. 23, p. 6279–6286, 2010.

MARTINS, E. A.; GERMANO, P. M. L. *Listeria monocytogenes* in ready-to-eat, sliced, cooked ham and salami products, marketed in the city of São Paulo, Brazil: Occurrence, quantification, and serotyping. Food Control, v. 22, n. 2, p. 297–302, 2011.

MAURY, M. M. *et al.* Hypervirulent *Listeria monocytogenes* clones' adaption to mammalian gut accounts for their association with dairy products. Nature Communications, v. 10, n. 1, p. 2488, 2019.

MAURY, M. M. *et al.* Uncovering *Listeria monocytogenes* hypervirulence by harnessing its biodiversity. Nature Genetics, v. 48, n. 3, p. 308, 2016.

MEIRELES, A. *et al.* The current knowledge on the application of anti-biofilm enzymes in the food industry. Food Research International, v. 86, p. 140–146, 2016.

MELONI, D. *et al. Listeria monocytogenes* in five Sardinian swine slaughterhouses: prevalence, serotype, and genotype characterization. Journal of Food Protection, v. 76, n. 11, p. 1863–1867, 2013.

MELONI, D. Presence of *Listeria monocytogenes* in Mediterranean-Style Dry Fermented Sausages. Foods, v. 4, n. 1, p. 34–50, 2015. Disponível em: <http://www.mdpi.com/2304-8158/4/1/34/>.

MEREGHETTI, L. *et al.* Low sensitivity of *Listeria monocytogenes* to quaternary ammonium compounds. Applied and Environmental Microbiology, v. 66, n. 11, p. 5083–5086, 2000.

METAXOPOULOS, J.; SAMELIS, J.; PAPADELLI, M. Technological and microbiological evaluation of traditional processes as modified for the industrial manufacturing of dry fermented sausage in Greece. Italian Journal of Food Science (Italy), 2001.

MEYER, C. *et al.* Occurrence of *L. monocytogenes* in ready-to-eat poultry products available on the German market. Food Research International, v. 48, n. 2, p. 944–947, 2012.

MIETTINEN, M. K; BJÖRKROTH, K. J.; KORKEALA, H. J. Characterization of *Listeria monocytogenes* from an ice cream plant by serotyping and pulsed-field gel electrophoresis. International Journal of Food Microbiology, v. 46, n. 3, p. 187–192, 1999.

MINEI, Claudia C *et al.* Influence of peroxyacetic acid and nisin and coculture with Enterococcus faecium on *Listeria monocytogenes* biofilm formation. Journal of Food Protection, v. 71, n. 3, p. 634–638, 2008.

MOLTZ, A. G.; MARTIN, S. E. Formation of biofilms by *Listeria monocytogenes* under various growth conditions. Journal of Food Protection, v. 68, n. 1, p. 92–97, 2005.

MOURA, A. *et al.* Whole genome-based population biology and epidemiological surveillance of *Listeria monocytogenes*. Nature Microbiology, v. 2, n. 2, p. 1–10, 2016. Disponível em: <http://dx.doi.org/10.1038/nmicrobiol.2016.185>.

NESBAKKEN, T.; KAPPERUD, G.; CAUGANT, D. A. Pathways of *Listeria monocytogenes* contamination in the meat processing industry. International Journal of Food Microbiology, v.

31, n. 1–3, p. 161–171, 1996.

NETSCHAJEW, A. *et al.* Detection of *Listeria monocytogenes* in vacuum-packed meat products using real-time PCR, immunoassay and culturing. Archiv für Lebensmittelhygiene, v. 60, n. 1, p. 12–17, 2009.

NIELSEN, E. M. *et al.* Closing gaps for performing a risk assessment on *Listeria monocytogenes* in ready-to-eat (RTE) foods: activity 3, the comparison of isolates from different compartments along the food chain, and from humans using whole genome sequencing (WGS) analysis. EFSA Supporting Publications, v. 14, n. 2, p. 1151E, 2017.

NØRRUNG, Birgit; ANDERSEN, Jens Kirk; SCHLUNDT, Jørgen. Incidence and control of *Listeria monocytogenes* in foods in Denmark. International Journal of Food Microbiology, v. 53, n. 2–3, p. 195–203, 1999.

NORWOOD, D. E.; GILMOUR, A. The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. Journal of applied microbiology, v. 88, n. 3, p. 512–520, 2000.

NOWAK, J. *et al.* Biofilm formation of *Listeria monocytogenes* 15G01, a persistent isolate from a seafood-processing plant in New Zealand, is influenced by inactivation of multiple genes belonging to different functional groups. Identification and understanding the roles of biofilm formation-related genes in *Listeria monocytogenes* isolated from seafood, p. 99, 2017.

O'DRISCOLL, B.; GAHAN, C. G; HILL, C. Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence. Applied and Environmental Microbiology, v. 62, n. 5, p. 1693–1698, 1996.

OLAIMAT, A. N. *et al.* Emergence of Antibiotic Resistance in *Listeria monocytogenes* Isolated from Food Products: A Comprehensive Review. *Comprehensive Reviews in Food* Science and Food Safety, v. 17, n. 5, p. 1277–1292, 2018.

OUYANG, Y. *et al.* Genome-wide screening of genes required for *Listeria monocytogenes* biofilm formation. Journal of Biotech Research, v. 4, p. 13, 2012.

OZBEY, G.; ERTAS, H. B.; KOK, F. Prevalence of *Listeria* species in camel sausages from retail markets in Aydin province in Turkey and RAPD analysis of *Listeria monocytogenes* isolates. Irish Veterinary Journal, v. 59, n. 6, p. 342, 2006.

OZBEY, G.; OZBEY, U.; KOK, F. Seasonal Prevalence of *Escherichia coli* O157: H7, *Listeria* spp. and *Salmonella* spp. in Fermented Sausages, Sosis and Salami in Eastern Turkey. Pakistan Veterinary Journal, v. 37, n. 3, p. 364–367, 2017.

PAN, Y.; BREIDT, F.; KATHARIOU, S. Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. Applied and Environmental Microbiology, v. 72, n. 12, p. 7711–7717, 2006.

PEANO, C. *et al.* Qualitative and quantitative evaluation of the genomic DNA extracted from GMO and non-GMO foodstuffs with four different extraction methods. Journal of Agricultural and Food Chemistry, v. 52, n. 23, p. 6962–6968, 2004.

PECCIO, A. *et al. Listeria monocytogenes* occurrence and characterization in meat-producing plants. Letters in Applied Microbiology, v. 37, n. 3, p. 234–238, 2003.

PÉREZ-RODRÍGUEZ, F. *et al.* Evaluation of hygiene practices and microbiological quality of cooked meat products during slicing and handling at retail. Meat Science, v. 86, n. 2, p. 479–485, 2010.

PETRUZZELLI, A. *et al.* Occurrence of *Listeria monocytogenes* in salami manufactured in the Marche Region (central Italy). Journal of Veterinary Medical Science, v. 72, n. 4, p. 499–502, 2010.

PHILLIPS, I. *et al.* Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. Journal of Antimicrobial Chemotherapy, v. 53, n. 1, p. 28–52, 2004.

PIERCEY, M. J.; HINGSTON, P. A.; HANSEN, L. T. Genes involved in *Listeria* *monocytogenes* biofilm formation at a simulated food processing plant temperature of 15 C. International Journal of Food Microbiology, v. 223, p. 63–74, 2016.

PIETA, L. *et al.* Transcriptional analysis of genes related to biofilm formation, stress-response, and virulence in *Listeria monocytogenes* strains grown at different temperatures. Annals of Microbiology, v. 64, n. 4, p. 1707–1714, 2014.

POIMENIDOU, S. V. *et al.* Variability of *Listeria monocytogenes* strains in biofilm formation on stainless steel and polystyrene materials and resistance to peracetic acid and quaternary ammonium compounds. International Journal of Food Microbiology, v. 237, p. 164–171, 2016.

PÖNTINEN, A. *et al.* Screening of the two-component-system histidine kinases of *Listeria monocytogenes* EGD-e. LiaS is needed for growth under heat, acid, alkali, osmotic, ethanol and oxidative stresses. Food Microbiology, v. 65, p. 36–43, 2017.

PÖNTINEN, A. *et al.* Two-component-system histidine kinases involved in growth of *Listeria monocytogenes* EGD-e at low temperatures. Applied and Environmental Microbiology, v. 81, n. 12, p. 3994–4004, 2015.

POURSHABAN, M. *et al.* Transferable tetracycline resistance in *Listeria monocytogenes* from food in Italy. Journal of Medical Microbiology, v. 51, n. 7, p. 564–597, 2002.

PRENCIPE, V. A. *et al. Listeria monocytogenes* prevalence, contamination levels and strains characterization throughout the Parma ham processing chain. Food Contro*l*, v. 25, n. 1, p. 150–158, 2012.

PROCESS, Sanitizing; FOOD, F O R; SURFACES, Contact. Allowed Detergents and Sanitizers for Food Contact Surfaces and Equipment in Organic Operations OVERVIEW : A TYPICAL CLEANING AND SANITIZING PROCESS FOR FOOD. p. 1–4, 2011.

PUGA, C. H; ORGAZ, B.; SANJOSE, C. *Listeria monocytogenes* impact on mature or old *Pseudomonas fluorescens* biofilms during growth at 4 and 20° C. *Frontiers in microbiology*, v. 7, p. 134, 2016.

QUILLIN, S. J.; SCHWARTZ, K. T.; LEBER, J. H. The novel *Listeria monocytogenes* bile sensor BrtA controls expression of the cholic acid efflux pump MdrT. *Molecular microbiology*, v. 81, n. 1, p. 129–142, 2011.

RABINOVICH, L. *et al.* Prophage excision activates *Listeria* competence genes that promote phagosomal escape and virulence. *Cell*, v. 150, n. 4, p. 792–802, 2012.

RAKIC-MARTINEZ, M. *et al. Listeria monocytogenes* strains selected on ciprofloxacin or the disinfectant benzalkonium chloride exhibit reduced susceptibility to ciprofloxacin, gentamicin, benzalkonium chloride, and other toxic compounds. Applied and Environmental Microbiology, v. 77, n. 24, p. 8714–8721, 2011.

REIJ, M. W.; DEN AANTREKKER, E. D.; FORCE, I. Europe Risk Analysis in Microbiology Task. Recontamination as a source of pathogens in processed foods. International Journal of Food Microbiology, v. 91, n. 1, p. 1–11, 2004.

ROBERTS, A. J. *et al.* Some *Listeria monocytogenes* outbreak strains demonstrate significantly reduced invasion, inlA transcript levels, and swarming motility in vitro. Applied and Environmental Microbiology, v. 75, n. 17, p. 5647–5658, 2009.

ROCHE, S. M. *et al.* Assessment of the virulence of *Listeria monocytogenes*: agreement between a plaque-forming assay with HT-29 cells and infection of immunocompetent mice. International Journal of Food Microbiology, v. 68, n. 1–2, p. 33–44, 2001.

RYU, J. H.; BEUCHAT, L. R. Biofilm formation and sporulation by *Bacillus cereus* on a stainless steel surface and subsequent resistance of vegetative cells and spores to chlorine, chlorine dioxide, and a peroxyacetic acid–based sanitizer. Journal of Food Protection, v. 68, n. 12, p. 2614–2622, 2005.

SAFDAR, A.; ARMSTRONG, D. Antimicrobial activities against 84 *Listeria monocytogenes* isolates from patients with systemic listeriosis at a comprehensive cancer center (1955-1997).

Journal of Clinical Microbiology, v. 41, n. 1, p. 483–485, 2003.

SALAMANO, R. *et al.* Neurolisteriosis in adults: report of six clinical cases. Arquivos de neuro-psiquiatria, v. 63, n. 4, p. 1063–1069, 2005.

SALUDES, M.; TRONCOSO, M.; FIGUEROA, G. Presence of *Listeria monocytogenes* in Chilean food matrices. Food Control, v. 50, p. 331–335, 2015.

SCHARFF, Robert L. Economic burden from health losses due to foodborne illness in the United States. Journal of Food Protection, v. 75, n. 1, p. 123–131, 2012.

SCHMID, B. *et al.* Role of cold shock proteins in growth of *Listeria monocytogenes* under cold and osmotic stress conditions. Applied and Environmenatl Microbiology, v. 75, n. 6, p. 1621–1627, 2009.

SCHUCHAT, A.; SWAMINATHAN, B.; BROOME, CLAIRE V. Epidemiology of human listeriosis. Clinical Microbiology Reviews, v. 4, n. 2, p. 169–183, 1991.

SERENO, M. J. *et al.* Distribution, adhesion, virulence and antibiotic resistance of persistent *Listeria monocytogenes* in a pig slaughterhouse in Brazil. Food Microbiology, 2019.

SERGELIDIS, D. *et al.* Temperature distribution and prevalence of *Listeria* spp. in domestic, retail and industrial refrigerators in Greece. International Journal of Food Microbiology, v. 34, n. 2, p. 171–177, 1997.

SILVA, D. A. L. *et al. Listeria* spp. contamination in a butcher shop environment and Listeria monocytogenes adhesion ability and sensitivity to food-contact surface sanitizers. Journal of Food Safety, v. 37, n. 2, p. e12313, 2017.

SIMÕES, M.; SIMOES, L. C.; VIEIRA, M. J. A review of current and emergent biofilm control strategies. LWT-Food Science and Technology, v. 43, n. 4, p. 573–583, 2010.

SKANDAMIS, P. N.; NYCHAS, G. J. E. Quorum sensing in the context of food microbiology. Applied and Environmental Microbiology, v. 78, n. 16, p. 5473–5482, 2012.

SKOVGAARD, N.; NØRRUNG, B.. The incidence of *Listeria* spp. in faeces of Danish pigs and in minced pork meat. International Journal of Food Microbiology, v. 8, n. 1, p. 59–63, 1989.

SOUMET, C. ; RAGIMBEAU, C. ; MARIS, P. Screening of benzalkonium chloride resistance in *Listeria monocytogenes* strains isolated during cold smoked fish production. Letters in Applied Microbiology, v. 41, n. 3, p. 291–296, 2005.

STEINER, A. E; MARAGOS, M. M.; BRADLEY JR, R L. Cleanability of stainless steel surfaces with various finishes. Dairy, Food and Environmental Sanitation, v. 20, n. 4, p. 250–260, 2000.

STEPANOVIĆ, S. *et al.* Biofilm formation by Salmonella spp. and *Listeria monocytogenes* on plastic surface. Letters in Applied Microbiology, v. 38, n. 5, p. 428–432, 2004.

STEPANOVIĆ, S. *et al.* Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by *staphylococci*. Apmis, v. 115, n. 8, p. 891–899, 2007.

STEWART, P. S.; FRANKLIN, M. J. Physiological heterogeneity in biofilms. Nature Reviews Microbiology, v. 6, n. 3, p. 199, 2008.

STOODLEY, P. *et al.* Biofilms as complex differentiated communities. Annual Reviews in Microbiology, v. 56, n. 1, p. 187–209, 2002.

SWAMINATHAN, B.; GERNER-SMIDT, P. The epidemiology of human listeriosis. Microbes and Infection, v. 9, n. 10, p. 1236–1243, 2007.

TAORMINA, P. J.; BEUCHAT, L. R. Survival of *Listeria monocytogenes* in commercial food-processing equipment cleaning solutions and subsequent sensitivity to sanitizers and heat. Journal of Applied Microbiology, v. 92, n. 1, p. 71–80, 2002.

THÉVENOT, D. *et al.* Fate of *Listeria monocytogenes* in experimentally contaminated French sausages. International Journal of Food Microbiology, v. 101, n. 1–2, p. 189–200, 2005a.

THÉVENOT, D. *et al.* Prevalence of *Listeria monocytogenes* in 13 dried sausage processing plants and their products. *I*nternational Journal of Food Microbiology, v. 102, n. 1, p. 85–94, 25 jun. 2005b. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/15925005>. Acesso em: 14 mar. 2018.

THÉVENOT, D. *et al.* Fate of *Listeria monocytogenes* in experimentally contaminated French sausages. International journal of food microbiology, v. 101, n. 2, p. 189–200, 2005c.

THÉVENOT, D.; DERNBURG, A.; VERNOZY-ROZAND, C. An updated review of *Listeria monocytogenes* in the pork meat industry and its products. Journal of Applied Microbiology, v. 101, n. 1, p. 7–17, 2006b.

TODHANAKASEM, T.; YOUNG, G. M. Loss of flagellum-based motility by *Listeria monocytogenes* results in formation of hyperbiofilms. Journal of bacteriology, v. 190, n. 17, p. 6030–6034, 2008.

TOMPKIN, R B. Control of *Listeria monocytogenes* in the food-processing environment. Journal of Food Protection, v. 65, n. 4, p. 709–725, 2002.

TRIMOULINARD, A. *et al.* Contamination by *Salmonella* spp., *Campylobacter* spp. and *Listeria* spp. of most popular chicken-and pork-sausages sold in Reunion Island. International journal of Food Microbiology, v. 250, p. 68–74, 2017.

TYÖPPÖNEN, S.; PETÄJÄ, E.; MATTILA-SANDHOLM, T. Bioprotectives and probiotics for dry sausages. International Journal of Food Microbiology, v. 83, n. 3, p. 233–244, 2003.

VALDERRAMA, W. B.; CUTTER, C. N. An ecological perspective of *Listeria monocytogenes* biofilms in food processing facilities. Critical Reviews in Food Science and Nutrition, v. 53, n. 8, p. 801–817, 2013.

VAN DEN ELZEN, A. M. G.; SNIJDERS, J. M. A. Critical points in meat production lines regarding the introduction of *Listeria monocytogenes*. Veterinary Quarterly, v. 15, n. 4, p. 143–145, 1993.

VASSEUR, C. *et al.* Combined effects of NaCl, NaOH, and biocides (monolaurin or lauric acid) on inactivation of *Listeria monocytogenes* and Pseudomonas spp. Journal of food protection, v. 64, n. 9, p. 1442–1445, 2001.

VATANYOOPAISARN, S. *et al.* Effect of flagella on initial attachment of *Listeria monocytogenes* to stainless steel. Applied and Environmental Microbiology, v. 66, n. 2, p. 860–863, 2000.

VAZ, C. S. L. Determinação da diversidade fenotípica e genotípica de *Salmonella* enterica subsp. enterica sorovar Enteritidis no Rio Grande do Sul. Acta Scientiae Veterinariae, v. 36, n. 1, p. 72–73, 2008.

VERGHESE, B. *et al.* comK prophage junction fragments as markers for *Listeria monocytogenes* genotypes unique to individual meat and poultry processing plants and a model for rapid niche-specific adaptation, biofilm formation, and persistence. Applied and Environmental Microbiology, v. 77, n. 10, p. 3279–3292, 2011.

VIALETTE, M. *et al.* Growths kinetics comparison of clinical and seafood *Listeria monocytogenes* isolates in acid and osmotic environment. International Journal of Food Microbiology, v. 82, n. 2, p. 121–131, 2003.

WEMEKAMP-KAMPHUIS, H. H. *et al.* Enhanced levels of cold shock proteins in *Listeria monocytogenes* LO28 upon exposure to low temperature and high hydrostatic pressure. Applied and Environmental Microbiology, v. 68, n. 2, p. 456–463, 2002.

WIEDMANN, M. *et al.* General Stress Transcription Factor ςB and Its Role in Acid Tolerance and Virulence of *Listeria monocytogenes*. Journal of Bacteriology, v. 180, n. 14, p. 3650–3656, 1998.

WINKELSTRÖTER, L. K. *et al.* Lactobacillus sakei 1 and its bacteriocin influence adhesion of *Listeria monocytogenes* on stainless steel surface. Food Control, v. 22, n. 8, p. 1404–1407, 2011.

WINKELSTRÖTER, L. K. *et al.* Unraveling microbial biofilms of importance for food microbiology. Microbial Ecology, v. 68, n. 1, p. 35–46, 2014.

WINKELSTRÖTER, L. K.; DE MARTINIS, E. C. P. Different methods to quantify *Listeria monocytogenes* biofilms cells showed different profile in their viability. Brazilian Journal of Microbiology, v. 46, n. 1, p. 231–235, 2015.

YANG, H. *et al.* Efficacy of sanitizing agents against *Listeria monocytogenes* biofilms on high-density polyethylene cutting board surfaces. Journal of Food Protection, v. 72, n. 5, p. 990–998, 2009.

YANG, L. *et al.* Combating biofilms. Pathogens and Disease, v. 65, n. 2, p. 146–157, 2012.

YOKOYAMA, E. *et al.* The marked increase of *Listeria monocytogenes* isolation from contents of swine cecum. Comparative Immunology, Microbiology and Infectious Diseases, v. 28, n. 4, p. 259–268, 2005.

YU, T.; JIANG, X. Prevalence and characterization of *Listeria monocytogenes* isolated from retail food in Henan, China. Food Control, v. 37, p. 228–231, 2014.

ZEEVI, M. Kaplan *et al.* *Listeria monocytogenes* multidrug resistance transporters and cyclic di-AMP, which contribute to type I interferon induction, play a role in cell wall stress. Journal of Bacteriology, v. 195, n. 23, p. 5250–5261, 2013.

ZETZMANN, M. *et al.* Characterization of the biofilm phenotype of a *Listeria monocytogenes* mutant deficient in agr peptide sensing. Microbiology Open, p. e826, 2019.

OBJECTIVES

The aim of this study was to track, by phenotypic and molecular techniques, the contamination by *Listeria monocytogenes* in pork production chains, in line with the international concept "From Farm to Fork". Just like accessing the adhesion features of selected isolates. Considering the main goal, specific objectives were:

- ✓ Identification of pathogenicity factors of *L. monocytogenes* isolates and characterization of serotypes.

- ✓ To analyze susceptibility to antibiotics of *L. monocytogenes* isolates.

- ✓ To evaluate the adhesion ability to polystyrene and biofilm formation of selected isolates in microplates and stainless steel coupons. And evaluate the effect of concentrations of salts and disinfectants under specific conditions of time and temperature, in the induction of stainless steel biofilms.

- ✓ To perform an expression predictive analysis of the genes related to biofilm formation and resistance to environmental stress (previously detected by genomic analysis) by qPCR assays.

CHAPTER 1. Occurrence of *Listeria monocytogenes* "from farm to fork" in a pork production chain

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RESUMO

No presente estudo, a contaminação por *L. monocytogenes* foi avaliada em diferentes etapas de uma cadeia de produção de suínos. Dez lotes de porcos foram amostrados em: baia de terminação, abate (após sangria, após chamuscamento, após evisceração, após lavagem final), processamento (facas, mesas de desossa, mão de manipuladores) e produtos finais (costelas, paleta, pernil e linguiça). Todas as amostras (n = 670) foram submetidas à detecção de *L. monocytogenes*, e os isolados obtidos (n = 18) foram analisados por suas características bioquímicas, sorogrupos, genes de virulência, perfis de PFGE, suscetibilidade a antibióticos (ampicilina, penicilina, gentamicina e sulfametoxazol / trimetoprim) e capacidade de adesão. Os resultados revelaram a baixa ocorrência de *Listeria* spp. na cadeia de produção de suínos avaliada. No entanto, quatro amostras de linguiça testadas (40%) foram positivas para *Listeria* spp., Sendo *L. monocytogenes* identificada em duas (20%). Dez isolados foram identificados como *L. monocytogenes* (oito de serogrounp 1/2a ou 3a e dois de sorogrupo 4b, 4d ou 4e), todos positivos para genes relacionados a virulência *hlyA*, *iap*, *plcA*, *actA*, i*nlA*, *inlB*, *inlC* e *inlJ*, e também suscetíveis a os antibióticos testados. Uma amostra de linguiça foi contaminada por ambos os sorogrupos 1/2a ou 3a e 4b, 4d ou 4e. Isolados do sorogrupo 1/2a ou 3a obtidos nas visitas 5 e 6 apresentaram perfis genéticos distintos por PFGE, sugerindo que a contaminação pode vir de fonte diferente. O potencial de adesão exibido por *Listeria* spp. isolados (n = 18) variaram de fraco (sorogrupo 4b, 4d ou 4e) a moderado (*L. innocua* e *L. monocytogenes* sorogrupo 1/2a ou 3a). Apesar da baixa ocorrência de *L. monocytogenes*, foram detectados sorogrupos patogênicos em linguiças, exigindo medidas de controle por parte da indústria.

Palavras-chave: *Listeria monocytogenes*, carne suína, cadeia produtiva

ABSTRACT

In the present study, *L. monocytogenes* contamination was assessed in different steps of a pork production chain. Ten lots of pigs were sampled at: termination barns, slaughtering (after bleeding, after buckling, after evisceration, after final washing), processing (knives, deboning tables, employee's hand) and end products (ribs, shoulder, ham and sausage). All samples (n= 670) were subjected to *L. monocytogenes* detection, and the obtained isolates (n= 18) were characterized by their biochemical characteristics, serogroups, virulence genes, PFGE profiles, antibiotic susceptibility (ampicillin, penicillin, gentamicin and sulfamethoxazole/trimethoprim) and adhesion abilities. The results revealed the low occurrence of *Listeria* spp. in the evaluated pork production chain. However, four tested sausage samples (40%) were positive for *Listeria* spp., being *L. monocytogenes* identified in two (20%). Ten isolates were identified as *L. monocytogenes* (eight from serogrounp 1/2a or 3a and two from serogroup 4b, 4d, or 4e), all positive for virulence related genes *hlyA*, *iap*, *plcA*, *actAinlA*, *inlB*, *inlC* and *inlJ* and susceptible to the tested antibiotics. One sausage sample was contaminated by both serogroup 1/2a or 3a and 4b, 4d, or 4e. Isolates from serogroup 1/2a or 3aobtained at visits 5 and 6 presented distinct genetic profiles by PFGE, suggesting that contamination may came from different source. The adhesion potential exhibited by *Listeria* spp. isolates (n=18) ranged from weak (serogroup 4b, 4d, or 4e) to moderate (*L. innocua* and *L. monocytogenes* serogroup 1/2a or 3a). Despite the low occurrence of *L. monocytogenes*, pathogenic serogroups were detected in sausages, demanding control measures by the industry.

Keywords: *Listeria monocytogenes*, pork meat, production chain

1 INTRODUCTION

Listeria monocytogenes is considered as an emerging foodborne pathogen, mainly due to the changing in human diet habits associated with modifications in the food production chain and the ability of this organism in surviving under adverse conditions (DONNELLY, 2001). Sporadic listeriosis cases and outbreaks have been associated with pork products, demonstrating their potential risks to consumers and demanding proper monitoring and control in the pork production chain (HELLSTRÖM *et al.*, 2010). *L. monocytogenes* was already isolated from different steps of the pork production chain, and the contamination sources are usually associated to the animals and the processing environment: this pathogen can successfully persist in biofilms in equipment and utensils, but it can occasionally be isolated from feces and skin from healthy carriers animals (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b).

The possible contamination routes throughout the pork production chain and factors that affect the presence of *L. monocytogenes* in this ecological niche need to be characterized, aiming to prevent the contamination in the end products available to consumers. However, there is little information in Brazil related to the contamination routes of *L. monocytogenes* in the entire pork production chain (SERENO *et al.*, 2019).

The monitoring of *L. monocytogenes* along the pork production chain is an important tool to ensure the safety of the end products to consumers, and also to provide relevant data related to the main contamination sources to guide proper control and sanitizing procedures (POWELL, 2013). Only based on data from the entire production chain it is possible to conduct further studies to investigate similarities between isolates obtained from infected animals, carcasses, processing environment and end products, allowing a deep characterization of the zoonotic/pathogenic potential of *L. monocytogenes* and its risks for consumers (HELLSTRÖM *et al.*, 2010). Besides the identification of the contamination sources, the pathogenicity of the *L. monocytogenes* isolates must be investigated through molecular and phenotypical methods, allowing the full characterization of this foodborne pathogen in the pork production chain and its potential impacts on human health (SERENO *et al.*, 2019).

This study aimed to identify the main contamination sources of contamination by *L. monocytogenes* in a pork production chain in Brazil, since pig farms to end products, and also to perform a characterization of the obtained isolates through their genetic, virulence, antibiotic susceptibility profiles and adhesion abilities.

2 MATERIAL AND METHODS

2.1 Sampling

The study was conducted in two full-cycle pig farms (farm 1 and farm 2) and a slaughterhouse located in the Minas Gerais state, Brazil, subjected to Federal Inspection Service from the Brazilian Ministry of Agriculture. Each farm was visited five times for sampling of the finishing barns of the pig lots (n = 10) that were sent to slaughtering in the following day. In the slaughterhouse, these same pig lots were sampled at different steps of slaughtering, since arrival to end products. Sampling points, units and sizes are described in Table 1.

The first stage of sampling took place in the pig farm where superficial samples were obtained from termination shed floor by overshoes method as described by Botteldoorn *et al.*, (2003). The second phase was carried out in the slaughterhouse where superficial samples of environment, carcass, equipment and utensils were collected. In the slaughtering room four points ([a]after bleeding, [b]afterbuckling, [c]after evisceration and [d]after final washing) were sampled by superficial swab of 400 cm^2. To ensure that the carcasses collected after bleeding were the same collected in subsequent steps, an appropriate pencil marking was done on the animals and collected at intervals of two pigs within the batch.

In the processing room the points sampled were: employee's hands, deboning table and knife, being these points sampled in two moments: before and during the activities. Finally, 25g units were obtained from packed end cuts (palette, rib and shank) and a packed sausage (fresh type sausage).

End products were collected under sterile conditions using sterile scalpels and packed in appropriate plastic bags (Nasco ™, Fort Atkinson, WI, USA). Surface samples were collected with appropriate sponges (3M Microbiology, St. Paul, MN, USA) for each type of sample (floors, carcasses, processing utensils). Surface samples (400 cm²) and overshoes were obtained by swabbing sterile sponges pre-moistened in 40 mL of 0.85% NaCl in four areas delimited by 10 x 10 cm (100 cm²) sterile plastic molds; specifically, in relation to the carcasses, the points sampled were the regions corresponding to the chest (front) and leg (back) on the medial and lateral sides of both hemi-carcasses (MARTINS *et al.*, 2018).

All the collected samples were conditioned in isothermal containers and kept under refrigeration until the moment of the laboratory analysis. Samples obtained from swabs, overshoes and final products received 160 mL, 160 mL and 225 mL of 0.85% peptone saline, respectively, and were homogenized (Stomacher 400 circulator, Seward Ltd., East Close, Sussex, UK) for 2 minutes. Since, 1 mL of the homogenate corresponds to 2 cm^2 of each sample obtained by swab and overshoes, and 0.1 g of final products.

2.2 *Listeria monocytogenes* detection

Samples were checked for the presence of *L. monocytogenes* according to ISO 11290-1 and ISO 11290-2 (ISO, E N, 1996; ISO, ISO, 2004). Aliquots of 40 mL of the sample suspensions were centrifuged, and the pellet was resuspended in half Fraser broth (Oxoid Ltd.) and incubated at 30 °C for 24 h. The obtained cultures were streaked onto *Listeria* chromogenic agar (Oxoid Ltd.) and *Listeria* selective base (Oxford, Oxoid Ltd.) and incubated at 37 °C for 48 h. Simultaneously, a 0.1 mL aliquot was transferred to tubes containing 10 mL of Fraser broth (Oxoid Ltd.) and incubated at 35 °C for 24 h. Then, the obtained cultures were streaked onto plates containing ALOA and Oxford agar and incubated at 37 °C for 48 h. The obtained colonies were subjected to biochemical tests for the production of catalase, motility at 25 °C, carbohydrates fermentation (dextrose, xylose, rhamnose, and mannitol), and hemolysis production on horse blood agar, for identification at species level.

2.3 Molecular Characterization

2.3.1 Genus / species PCR confirmation

After biochemical characterization, the identified isolates were subjected to DNA extraction and purification with the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA). The DNA extracted from each isolate was subjetced to a genus/species confirmatory PCR assay according to Hudson *et al.*, (2001). Amplification conditions were as described by the authors and the amplification mixture composed 12.5 µl of GoTaq Green Master Mix (Promega), 5 µL DNA, 5 µM of each primer 23S rRNA and *hly* (all primers sequences used in this work are showed in supplementary material (Table S1) and ultra pure PCR water (Promega) yielding a final reaction of 25 µl. Five microliters aliquots of PCR products were electrophoresed on 2.0% (m/v) agarose gels and 0.5 M Tris/borate/EDTA buffer (TBE) stained with GelRed (Biotium Inc., Hayward, CA, USA), and visualized on a transilluminator. For each target DNA region, the following PCR product sizes were observed: 239 bp for 23S rRNA and 706 bp for *hly*. In all molecular assays, the strains *L. monocytogenes* Scott A, ATCC 7644, were used as positive control.

2.3.2 Serogrouping and Virulence PCR

The DNA extracted from each isolate was submitted to serogroup characterization according two protocols, Borucki & Call, (2003) and Doumith *et al.*, (2004). The isolates were further subjected to Simplex and Multiplex PCR reactions, proposed by Liu *et al.*, (2007) and Rawool *et al.*, (2007), to investigate virulence-related genes involved in cell invasion processes and host cell-to-cell spread. The reactions and amplification conditions were those described by the authors. Primers sequences are shown in Table S1.

2.3.3 Pulsed-field gel electrophoresis (PFGE)

The genomic relatedness of *L. monocytogenes* isolates was investigated by macrorestriction analysis with *Apa*I and *Asc*I endonucleases (Promega, Wisconsin, USA). For this, the standardized laboratory protocol for *L. monocytogenes* of PulseNet was followed (GRAVES; SWAMINATHAN, 2001). The DNA fragments were separated by PFGE using the CHEF DR II system (Bio-Rad, Hercules, USA) at 6.0 V/cm with 0.5X TBE as the running buffer. Whole cell DNA of *Salmonella* Branderup H9812 digested with *Xba*I endonuclease served as size marker. Macrorestriction patterns were analyzed using the BioNumerics software (Applied Maths, Sint-Martens Latem, Belgium), and the similarities between the patterns were compared based on the Dice correlation coefficient, with a maximal position tolerance of 1.5%. Patterns were clustered according to the unweighted pair group method with arithmetic averages (UPGMA). Fragments smaller than 20 kb were not considered for cluster analysis.

2.4 *Listeria* spp. adhesion potential

According to the recommendations of Djordjevic *et al*., (2002) and Larivière-Gauthier *et al.*, (2014), *Listeria* spp. isolates were subjected to adhesion capacity tests. Briefly, 20 µL of an overnight culture of *L. monocytogenes*was transferred to a well of a microtiter plate containing 130 µL of TSB (Oxoid) and incubatedat 37 °C for 24 h. After incubation, the cultures were discarded, and the wells were washed with PBS (pH 7.2) three times to remove non-adhered cells. The cells were fixed by addition of methanol (Sigma- Aldrich), and air-dried for 10 min. Then, crystal violet solution (1% w/v) was added, and after 15 min the plates were washed with water. After drying, 95% ethanol was added to each well, and the absorbance was measured after incubation for 30 min at room temperature (λ =595 nm, Multi Scan FC, (Thermo Fisher Scientific, Waltham, EUA). All tests were performed with four replicates.

The results of the adhesion test were evaluated according to the classification proposed by Stepanović *et al.*, (2007). For each isolate, the mean values of the recorded optical density were calculated (OD isolate), and compared with the adjusted OD value from the blank wells ($OD_{ad\text{-}bl}$) that were calculated as the following:

$OD_{ad\text{-}bl}$ = average of blank OD + (3 x standard deviation of blank OD)

Based on the comparisons, each isolate was characterized accordingto the following classification:

Absence of adhesion OD isolate $\leq OD_{ad\text{-}bl}$
Weak adhesion $OD_{ad\text{-}bl} <$ OD isolate $\leq 2 \times OD_{ad\text{-}bl}$
Moderate adhesion $2 \times OD_{ad\text{-}bl} <$ OD isolate $\leq 4 \times OD_{ad\text{-}bl}$
Strong adhesion OD isolate $> 4 \times OD_{ad\text{-}bl}$

2.5 Broth microdilution for antimicrobial susceptibility testing

The isolates were submitted to the minimum inhibitory concentration (MIC) test for ampicillin, penicillin, gentamicin and sulfamethoxazole / trimethoprim, as determined for *L. monocytogenes* (CLSI, 2006), using the broth microdilution method for tests of antimicrobial susceptibility (CLSI, 2012). *S. aureus* ATCC 29213 was used as a control and the results were interpreted according to previously established breakpoint values, classifying the isolates as sensitive or resistant (CLSI, 2017). The antimicrobial agents tested were selected based on the classes described by CLSI (2012) and EUCAST (2017).

3 RESULTS AND DISCUSSION

Studies aiming to detect foodborne pathogens in the entire food production chain (from farm to fork) are useful to identify specific steps that may play a role in food contamination. Here, among 760 samples collected (in 10 visits) along pork production chain, we identified *Listeria* spp. in only 4 (0.6%) samples, being only 2 (0.3%) positive for *L.*

monocytogenes (Table 1). Although the sampling was carried out in farms, different slaughtering steps, food processing environment, and final products, only sausages were contaminated with *L. innocua* (one sample collected in the visit 3 and another from visit 4) and *L. monocytogenes* (one sample collected in the visit 5 and another from visit 6). All obtained isolates (n=18) were subjected to PCR as described by Hudson *et al.*, (2001) and previous biochemical results were confirmed (Table S2), 8 isolates were identified as *L. innocua* and 10 as *L. monocytogenes*.

L.monocytogenes is occasionally isolated in pork production farms, from production environment, feces and skin of apparently healthy animal (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b). In the present study, the occurrence of *Listeria* spp. was accessed in two pork production farms in Minas Gerais State, Brazil, in which no sample were positive. The sampled lots were also submitted to carcass sampling at four steps during slaughtering and again all were negative. Similar as observed in the present study, Prencipe *et al.*, (2012) identified a low occurrence of *L. monocytogenes* on pork carcass (3%, 23/774) after slaughtering (before refrigeration).

Proper methods applied during slaughtering may limit *L. monocytogenes* prevalence in carcasses (KURPAS; WIECZOREK; OSEK, 2018). Kanuganti *et al.*, (2002b), observed that *L. monocytogenes* contamination was lower in carcass after slaughter when compared to the subsequently minced meat. The chilling and cutting stages were reported to significantly increase pork contamination, and a high pathogen environmental prevalence was observed in the chilling areas. There are also reports where *L. monocytogenes* was detected in minced meat from a negative carcass, suggesting that meat contamination occurred during meat processing through cross-contamination (KURPAS; WIECZOREK; OSEK, 2018).

These findings indicate that actions after slaughtering play a major role for meat contamination (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b). During the processing, meat products can be contaminated by raw meat, utensils, equipment, environmental surfaces, and food handling (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006a). In this study, *L. monocytogenes* was not detected in samples obtained in the processing environment (knife, hands of employees and deboning table), demonstrating the good practices of hygiene and sanitization applied by the industry. However, we detected *Listeria* spp. in final products at low frequencies: 5.7% (4/70), being 2.85% (2/70) contaminated by *L. monocytogenes*. Among the products sampled, only sausage presented positive results for *Listeria* spp.: 40% (4/10).

In a study that evaluated *L. monocytogenes* presence in fresh mixed sausage in Rio Grande Sul state, Brazil, Padilha da Silva *et al.*, (2004) found *L. monocytogenes* in 29.4% of the raw material samples used for sausages products (33.3% of the beef samples, 33.3% of the pork samples and 20% of the swine fat samples). Sausage is a popular pork product in Brazil, and some people may eat this product undercooked. Myasaki*et al.*, (2009) evaluated the *L. monocytogenes* prevalence in sausage samples collected at retail stores in São Paulo, Brazil, and obtained 90 % of the samples positive for *Listeria* spp.: *L. monocytogenes* was detected in 42% of the samples, with counts below 10^2 CFU/g. In Italy, (PECCIO *et al.*, 2003) reported that raw sausages are the product most frequently contaminated by *L. monocytogenes*.

The isolates (n=18) recovered in the present study were subjected to serogrouping by a PCR assay according to Borucki & Call (2003) and to Doumith *et al.*, (2004). The results obtained by both protocols were corresponded, *L. monocytogenes* isolates were classified as belonging to serogroups 1/2a or 3/a (n=8) and 4b/4d or 4e (n=2). As expected, all *L. monocytogenes* isolates harbored the virulence genes *inlA, inlB, inlC, inlJ, plcA, hlyA, actA,* and *iap*.

In this work, 10 *L. monocytogenes* isolates obtained from two sausage samples were identified as belonging to serogroups 1/2a or 3/a (n=8) and 4b, 4d or 4e (n=2). These serogroups are the major causative of human listeriosis (LOMONACO; NUCERA; FILIPELLO, 2015). Virulence potential is an important factor involved in the complex events that lead to disease (TOMPKIN, 2002). Certain *L. monocytogenes* strains are more virulent and much more likely to be involved in foodborne diseases than others. As expected, all isolates obtained from sausages in the present study harbored the virulence genes *inlA, inlB, inlC, inlJ, plcA, hlyA, actA*, and *iap*, similar as observed in others isolates obtained from other sources in Minas Gerais state, Brazil (CAMARGO *et al.*, 2014; DA SILVA *et al.*, 2016). These genes play an important role in the virulence mechanisms of this pathogen, and they are enrolled in different stages of pathogenesis, since cell invasion to cell-to-cell spread (CABANES *et al.*, 2002).

Many studies have documented *L. monocytogenes* contamination in a variety of pork products worldwide (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b). Discrimination between pathogenic and non-pathogenic strains is an important aspect to evaluate the microorganism significance towards food safety and public health (JENSEN *et al.*, 2008; ROBERTS *et al.*, 2009). The most frequent serotypes found in carcasses and slaughterhouses environment are 1/2a and 1/2c, while serotype 1/2b and 4b are usually found at low prevalence rates (MELONI, 2015; MELONI *et al.*, 2013; THÉVENOT *et al.*, 2005).

Regarding the antimicrobial susceptibility, all isolates (10 *L. monocytogenes* and 8 *L. innocua*) were sensitive to the four tested antibiotics (Table S3). In the last years, studies have demonstrated that antibiotic resistance has increase amongst *L. monocytogenes* obtained from processing environments and food (MAUNG *et al.*, 2019; OLAIMAT *et al.*, 2018; SERENO *et al.*, 2019), which is causing a great concern for public health. *L. monocytogenes* is usually sensitive to most antibiotics used against Gram positive bacteria and most common treatment involves β-lactam antibiotics (penicillin or ampicillin), potentially in conjunction with gentamicin. Trimethoprim/sulfamethoxazole can be used as alternative drugs for patients allergic to penicillin. But the therapeutic protocol and antibiotic choice varies according to the infection symptoms and the patient risk group (CAMARGO *et al.*, 2017; ROBERTS *et al.*, 2009; SWAMINATHAN; GERNER-SMIDT, 2007).

As expected, the phylogenetic lineages characterized by serogrouping PCR were grouped into different clusters in the macrorestriction analysis with ApaI and AscI endonucleases. PFGE analysis grouped the *Listeria* spp. isolates in five clusters (Figure 1). The first cluster was composed by *L. innocua* strains obtained in the visit 4 while the second cluster was composed by a single *L. innocua* recovered in the visit 3. The third and fourth clusters grouped *L. monocytogenes* serogroup 1/2a or 3a, where each one grouped isolates from visits 5 and 6, respectively, suggesting they came from a different source. The fifth cluster consists of two *L. monocytogenes* isolates serogroup 4b, 4d or 4e also obtained in the visit 6. Interestingly, a single sample in collect during visit 6 was contaminated by two different pathogenic serogroups.

Serogroup 1/2a or 3a is often associated with meat environment contamination, being well recognized by its ability to persist in this such environment (MAURY *et al.*, 2016). Thus the adhesion potential of all isolates obtained in this study was characterized (Table S4), and the results ranged from weak to moderate adhesion potential. Isolates from serogroup 1/2a or 3a presented a higher adhesion ability when compared to isolates 4b, 4d or 4e, according to previous studies (GALVÃO *et al.*, 2012; SERENO *et al.*, 2019).

Studies focused on the entire food production chain (from farm to fork) enables detection of foodborne pathogens contamination routes, allowing proper control and adoption of preventive measures. Here we carried out a complete study, where pork production farms, slaughtering, processing environment and final products made with pork meat were sampled and tested for the presence of the major pathogen *L. monocytogenes*. The low occurrence of *L. monocytogenes* observed in the entire pork production chain indicates that good practices are applied during pork production. However, sausages samples were contaminated by *Listeria* spp. and pathogenic *L. monocytogenes* serotypes, demanding control measure by the industry. Other studies have already demonstrated a high occurrence of *L. monocytogenes* in sausages in Brazil (MIYASAKI *et al.*, 2009; PADILHA DA SILVA *et al.*, 2004), so we emphasize the need of proper handling this product at home to avoid cross-contamination in the domestic environment as well as adequate cooking before consumption.

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Bibliography

BORUCKI, M. K.; CALL, D. R. *Listeria monocytogenes* serotype identification by PCR. Journal of Clinical Microbiology, v. 41, n. 12, p. 5537–5540, 2003.

BOTTELDOORN, N. *et al. Salmonella* on pig carcasses: positive pigs and cross contamination in the slaughterhouse. Journal of Applied Microbiology, v. 95, n. 5, p. 891–903, 2003.

CABANES, D. *et al.* Surface proteins and the pathogenic potential of *Listeria monocytogenes*. Trends in Microbiology, v. 10, n. 5, p. 238–245, 2002.

CAMARGO, A. C. *et al. Listeria monocytogenes* in Food-Processing Facilities, Food Contamination, and Human Listeriosis: The Brazilian Scenario. Foodborne Pathogens and Disease, v. 14, n. 11, p. fpd.2016.2274, 2017. Disponível em: <http://online.liebertpub.com/doi/10.1089/fpd.2016.2274>.

CAMARGO, A. C. *et al.* Low occurrence of *Listeria monocytogenes* on bovine hides and carcasses in Minas Gerais State, Brazil: molecular characterization and antimicrobial resistance. Journal of Food Protection, v. 77, n. 7, p. 1148–1152, 2014.

CLSI – Clinical and Laboratory Standards Institute. Methods for Antimicrobial
Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. Clinical and Laboratory Standards Institute CLSI document M45-A, 2006.

CLSI – Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, ninth ed. Clinical and Laboratory Standards Institute CLSI document M07-A9, 2012.

CLSI – Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing, 27th ed. Clinical and Laboratory Standards Institute CLSI supplement M100, 2017.

DA SILVA, D. A. L. *et al.* Hygiene and Safety in the Meat Processing Environment from Butcher Shops: Microbiological Contamination and *Listeria monocytogenes*. Journal of Food Protection, v. 79, n. 4, p. 628–634, 2016.

DE VALK, H. *et al.* Two consecutive nationwide outbreaks of listeriosis in France, October 1999–February 2000. American Journal of Epidemiology, v. 154, n. 10, p. 944–950, 2001.

DJORDJEVIC, D.; WIEDMANN, M.; MCLANDSBOROUGH, L. A. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Applied and Environmental Microbiology, v. 68, n. 6, p. 2950–2958, 2002.

DONNELLY, C. W. *Listeria monocytogenes*: a continuing challenge. Nutrition Reviews, v. 59, n. 6, p. 183–194, 2001.

DOUMITH, M. *et al.* Differentiation of the Major *Listeria monocytogenes* Serovars by Multiplex PCR Differentiation of the Major *Listeria monocytogenes* Serovars by Multiplex PCR. Journal of Clinical Microbiology, v. 42, n. 8, p. 3819–3822, 2004.

European Committee on Antimicrobial Susceptibility. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.1, 2017. *URL:* http://www. eucast. org/clinical_breakpoints/(Accessed 10/05/2018), 2017.

GALVÃO, N. N. *et al.* PFGE characterisation and adhesion ability of *Listeria monocytogenes* isolates obtained from bovine carcasses and beef processing facilities. Meat Science, v. 92, n. 4, p. 635–643, 2012.

GOULET, V. *et al.* Listeriosis outbreak associated with the consumption of rillettes in France in 1993. Journal of Infectious Diseases, v. 177, n. 1, p. 155–160, 1998.

GRAVES, L. M.; SWAMINATHAN, B. PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. International Journal of Food Microbiology, v. 65, n. 1, p. 55–62, 2001.

HELLSTRÖM, S. *et al. Listeria monocytogenes* Contamination in Pork Can Originate from Farms. Journal of Food Protection, v. 73, n. 4, p. 641–648, 2010. Disponível em: <http://jfoodprotection.org/doi/abs/10.4315/0362-028X-73.4.641>.

HOF, H.; ROCOURT, J.. Is any strain of *Listeria monocytogenes* detected in food a health risk? International Journal of Food Microbiology, v. 16, n. 3, p. 173–182, 1992.

HUDSON, J. A. *et al.* Rapid detection of *Listeria monocytogenes* in ham samples using immunomagnetic separation followed by polymerase chain reaction. Journal of Applied Microbiology, v. 90, n. 4, p. 614–621, 2001.

ISO, E N. 11290-1: Microbiology of food and animal feeding stuffs—horizontal method for the detection and enumeration of *Listeria monocytogenes*-Part 1: detection method. Geneva: International Organization for Standardization, 1996.

ISO, I S O. 11290-1/Amd 1—Modification of the Isolation Media and the Haemolysis Test, and Inclusion of Precision Data. Geneva: ISO, 2004.

JANAKIRAMAN, V. Listeriosis in pregnancy: diagnosis, treatment, and prevention. Reviews in Obstetrics and Gynecology, v. 1, n. 4, p. 179, 2008.

JENSEN, A. *et al.* Processing plant persistent strains of *Listeria monocytogenes* appear to have a lower virulence potential than clinical strains in selected virulence models. International Journal of Food Microbiology, v. 123, n. 3, p. 254–261, 2008.

KANUGANTI, S. R. *et al.* Detection of *Listeria monocytogenes* in pigs and pork. Journal of Food Protection, v. 65, n. 9, p. 1470–1474, 2002.

KURPAS, M.; WIECZOREK, Kinga; OSEK, Jacek. Ready-to-eat meat products as a source of *Listeria monocytogenes*. Journal of Veterinary Research, v. 62, n. 1, p. 49–55, 2018.

LARIVIÈRE-GAUTHIER, G. *et al.* Analysis of *Listeria monocytogenes* strain distribution in a pork slaughter and cutting plant in the province of Quebec. Journal of Food Protection, v. 77, n. 12, p. 2121–2128, 2014.

LIU, D. *et al.* A multiplex PCR for species-and virulence-specific determination of *Listeria monocytogenes*. Journal of Microbiological Methods, v. 71, n. 2, p. 133–140, 2007.

LOMONACO, S.; NUCERA, D.; FILIPELLO, V. The evolution and epidemiology of *Listeria monocytogenes* in Europe and the United States. Infection, Genetics and Evolution, v. 35, p. 172–183, 2015.

MARTINS, B. T. F. *et al. Yersinia enterocolitica* in a Brazilian pork production chain: Tracking of contamination routes, virulence and antimicrobial resistance. International Journal of food Microbiology, v. 276, p. 5–9, 2018.

MAUNG, A. T. *et al.* Antimicrobial resistance profiles of *Listeria monocytogenes* isolated from chicken meat in Fukuoka, Japan. International Journal of Food Microbiology, v. 304, p. 49–57, 2019.

MAURY, M. M. *et al.* Uncovering *Listeria monocytogenes* hypervirulence by harnessing its biodiversity. Nature Genetics, v. 48, n. 3, p. 308, 2016.

MELONI, D. *et al. Listeria monocytogenes* in five Sardinian swine slaughterhouses: prevalence, serotype, and genotype characterization. Journal of Food Protection, v. 76, n. 11, p. 1863–1867, 2013.

MELONI, D. Presence of *Listeria monocytogenes* in Mediterranean-Style Dry Fermented Sausages. Foods, v. 4, n. 1, p. 34–50, 2015. Disponível em: <http://www.mdpi.com/2304-8158/4/1/34/>.

MIYASAKI, K. N. *et al.* High prevalence, low counts and uncommon serotypes of *Listeria monocytogenes* in linguiça, a Brazilian fresh pork sausage. Meat Science, v. 83, n. 3, p. 523–527, 2009.

OLAIMAT, A. N. *et al.* Emergence of Antibiotic Resistance in *Listeria monocytogenes* Isolated from Food Products: A Comprehensive Review. Comprehensive Reviews in Food Science and Food Safety, v. 17, n. 5, p. 1277–1292, 2018.

PADILHA DA SILVA, W. *et al. Listeria* spp . no processamento de lingüiça frescal em frigoríficos de Pelotas , RS , Brasil. Ciência Rural, v. 34, n. 3, p. 911–916, 2004.

PECCIO, A. *et al. Listeria monocytogenes* occurrence and characterization in meat producing plants. Letters in Applied Microbiology, v. 37, n. 3, p. 234–238, 2003.

POWELL, M. R. How to model a negligible probability under the WTO sanitary and phytosanitary agreement? Risk Analysis, v. 33, n. 6, p. 972–983, 2013.

PRENCIPE, V. A. *et al. Listeria monocytogenes* prevalence, contamination levels and strains characterization throughout the Parma ham processing chain. Food Control, v. 25, n. 1, p. 150–158, 2012.

RAWOOL, D. B. *et al.* A multiplex PCR for detection of virulence associated genes in *Listeria monocytogenes*. International Journal of Food Safety, v. 9, p. 56–62, 2007.

ROBERTS, A. J. *et al.* Some *Listeria monocytogenes* outbreak strains demonstrate significantly reduced invasion, inlA transcript levels, and swarming motility in vitro. Applied and environmental microbiology, v. 75, n. 17, p. 5647–5658, 2009.

SALAMANO, R. *et al.* Neurolisteriosis in adults: report of six clinical cases. Arquivos de neuro-psiquiatria, v. 63, n. 4, p. 1063–1069, 2005.

SERENO, M. J. *et al.* Distribution, adhesion, virulence and antibiotic resistance of persistent *Listeria monocytogenes* in a pig slaughterhouse in Brazil. Food Microbiology, 2019.

STEPANOVIĆ, S. *et al.* Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. Apmis, v. 115, n. 8, p. 891–899, 2007.

SWAMINATHAN, B.; GERNER-SMIDT, P. The epidemiology of human listeriosis. Microbes and Infection, v. 9, n. 10, p. 1236–1243, 2007.

THÉVENOT, D. *et al.* Prevalence of *Listeria monocytogenes* in 13 dried sausage processing plants and their products. International Journal of Food Microbiology, v. 102, n. 1, p. 85–94, 25 jun. 2005. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/15925005>. Acesso em: 14 mar. 2017.

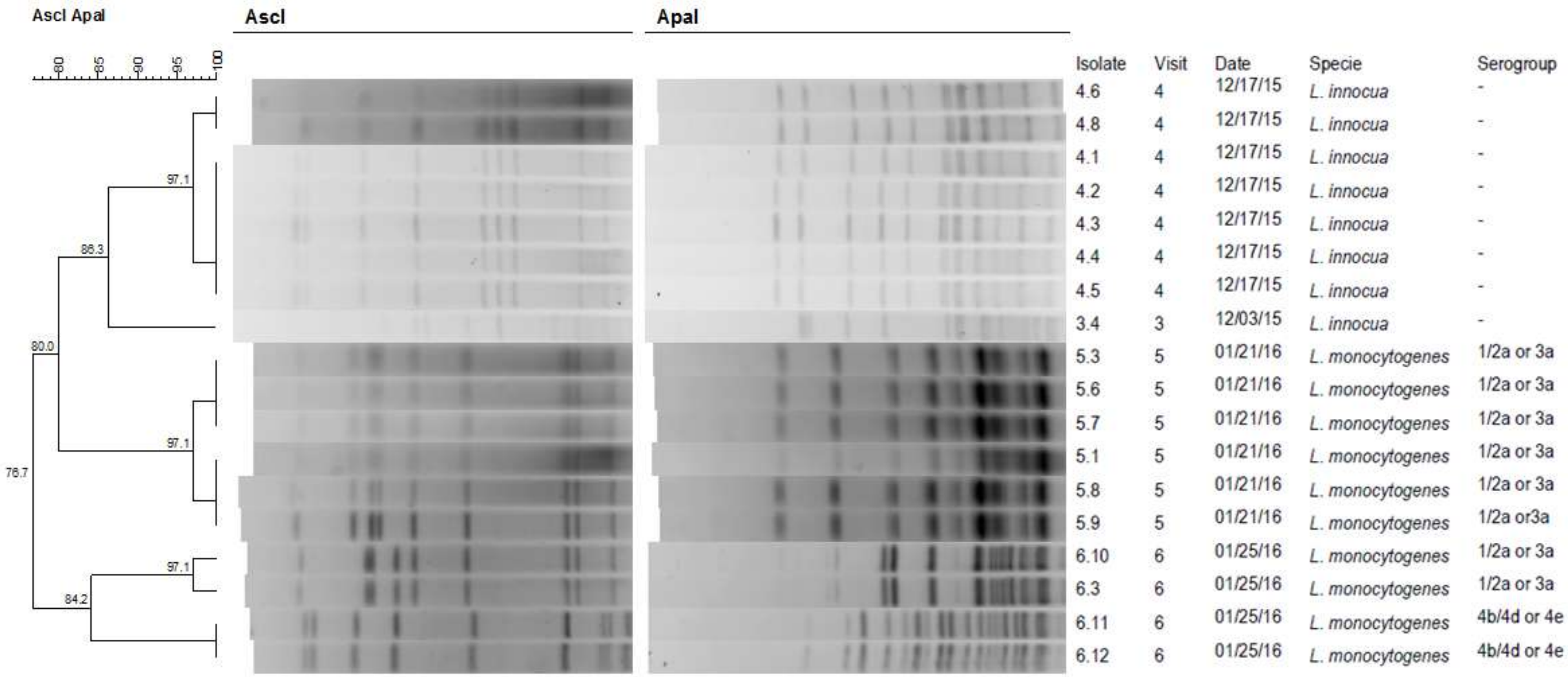
THÉVENOT, D.; DERNBURG., A.; VERNOZY-ROZAND, C. An updated review of *Listeria monocytogenes* in the pork meat industry and its products. Journal of Applied Microbiology, v. 101, n. 1, p. 7–17, 2006b.

TOMPKIN, R. B. Control of *Listeria monocytogenes* in the food-processing environment. Journal of Food Protection, v. 65, n. 4, p. 709–725, 2002.

Table 1. Occurrence of *Listeria* spp. and *L. monocytogenes* “from farm to fork" in a pork production chain.

Environment	Sample	Detail	Procedure	n	Positive samples (%)	
					Listeria spp.	*L. monocytogenes*
Farmproduction	Barn	-	Swab (400 cm^2)	20	0	0
Slaughtering	Carcass	A: after bleeding	Swab (400 cm^2)	100	0	0
		B: after singeing	Swab (400 cm^2)	100	0	0
		C: after evisceration	Swab (400 cm^2)	100	0	0
		D: after final wash	Swab (400 cm^2)	100	0	0
Processing	Knife	Before processing	Swab (400 cm^2)	30	0	0
		During processing	Swab (400 cm^2)	30	0	0
	Employee's hands	Before processing	Swab (400 cm^2)	30	0	0
		During processing	Swab (400 cm^2)	30	0	0
	Boningtable	Before processing	Swab (400 cm^2)	30	0	0
		During processing	Swab (400 cm^2)	30	0	0
	Final products	Ribs	Portions (25g)	20	0	0
		Shoulder	Portions (25g)	20	0	0
		Ham	Portions (25g)	20	0	0
		Sausage	Portions (25g)	10	4 (40%)	2 (20%)

Figure1. Dendogram of *Listeria monocytogenes* isolates based on *ApaI* and *AscI* macrorestriction patterns. The similarity analysis was performed using the Dice coefficient and UPGMA method (tolerance 1%). The sampling visits, specie and serogrouping identification are shown.



Isolate	Visit	Date	Specie	Serogroup
4.6	4	12/17/15	*L. innocua*	-
4.8	4	12/17/15	*L. innocua*	-
4.1	4	12/17/15	*L. innocua*	-
4.2	4	12/17/15	*L. innocua*	-
4.3	4	12/17/15	*L. innocua*	-
4.4	4	12/17/15	*L. innocua*	-
4.5	4	12/17/15	*L. innocua*	-
3.4	3	12/03/15	*L. innocua*	-
5.3	5	01/21/16	*L. monocytogenes*	1/2a or 3a
5.6	5	01/21/16	*L. monocytogenes*	1/2a or 3a
5.7	5	01/21/16	*L. monocytogenes*	1/2a or 3a
5.1	5	01/21/16	*L. monocytogenes*	1/2a or 3a
5.8	5	01/21/16	*L. monocytogenes*	1/2a or 3a
5.9	5	01/21/16	*L. monocytogenes*	1/2a or3a
6.10	6	01/25/16	*L. monocytogenes*	1/2a or 3a
6.3	6	01/25/16	*L. monocytogenes*	1/2a or 3a
6.11	6	01/25/16	*L. monocytogenes*	4b/4d or 4e
6.12	6	01/25/16	*L. monocytogenes*	4b/4d or 4e

SUPPLEMENTARY MATERIAL

Table S1. Sequence primers for each target gene in PCR screening.

Prìmer	Sequence	Size	Reference
23S rRNA	(F: GGGGAACCCACTATCTTTAGTC; R: GGGCCTTTCCAGACCGCTTCA)	239 bp	Hudson *et al*., (2001)
hly	(F: GCCTGCAAGTCCTAAGACGCCAATC R: CTTGCAACTGCTCTTTAGTAACAGC)	706 bp	Hudson *et al*., (2001)
D1	(F: CGATATTTTATCTACTTTGTCA; R: TTGCTCCAAAGCAGGGCAT)	214 bp	Borucki & Call (2003)
D2	(F: GCGGAGAAAGCTATCGCA; R: TTGTTCAAACATAGGGTA)	140 bp	Borucki & Call (2003)
GLT	(F: AAAGTGAGTTCTTACGAGATT; R: AATTAGGAAATCGACCTTCT)	483 bp	Borucki & Call (2003)
Lmo1118	(F: AGGGGTCTTAAATCCTGGAA R: CGGCTTGTTCGGCATACTTA)	906 bp	Doumith *et al*., (2004)
Lmo0737	(F: AGGGCTTCAAGGACTTACCC R: ACGATTTCTGCTTGCCATTC)	691 bp	Doumith *et al*., (2004)
ORF2110	(F: AGTGGACAATTGATTGGTGAA R: CATCCATCCCTTACTTTGGAC)	597 bp	Doumith *et al*., (2004)
ORF2819	(F: AGCAAAATGCCAAAACTCGT R: CATCACTAAAGCCTCCCATTG)	491 bp	Doumith *et al*., (2004)
prs	(F: GCTGAAGAGATTGCGAAAGAAG R: CAAAGAAACCTTGGATTTGCGG)	370 bp	Doumith *et al*., (2004)
inlA	(F: ACGAGTAACGGGACAAATGC; R: CCCGACAGTGGTGCTAGATT)	800 bp	Liu *et al*., (2007)
inlB	(F: TGGGAGAGTAACCCAACCAC; R: GTTGACCTTCGATGGTTGCT)	884 bp	Liu *et al*., (2007)
inlC	(F: AATTCCCACAGGACACAACC; R: CGGGAATGCAATTTTTCACTA)	517 bp	Liu *et al*., (2007)
inlJ	(F: GTAACCCCGCTTACACAGTT; R: AGCGGCTTGGCAGTCTAATA)	238 bp	Liu *et al*., (2007)
hlyA	(F: GCAGTTGCAAGCGCTTGGAGTGAA; R: GCAACGTATCCTCCAGAGTGATCG)	456 bp	Rawool *et al*., (2007)
actA	(F: CGCCGCGGAAATTAAAAAAAGA R: ACGAAGGAACCGGGCTGCTAG)	839 bp	Rawool *et al*., (2007)
Iap	(F: ACAAGCTGCACCTGTTGCAG; R: TGACAGCGTGTGTAGTAGCA)	131 bp	Rawool *et al*., (2007)
plcA	(F: CTGCTTGAGCGTTCATGTCTCATCCCCC; R: ATGGGTTTCACTCTCCTTCTAC)	1484 bp	Rawool *et al*., (2007)

Table S2. Genus/species confirmation PCR according to Hudson *et al*., (2001).

Genus/species confirmation PCR				Hudson *et al*., (2001)	
				Listeria-especific primer	*L. monocytogenes* -especific primer
Isolate	Sample	Visit	Date	23S rRNA	*Hly*
3.4	Sausage	3	12/03/15	+	-
4.1	Sausage	4	12/17/15	+	-
4.2	Sausage	4	12/17/15	+	-
4.3	Sausage	4	12/17/15	+	-
4.4	Sausage	4	12/17/15	+	-
4.5	Sausage	4	12/17/15	+	-
4.6	Sausage	4	12/17/15	+	-
4.8	Sausage	4	12/17/15	+	-
5.1	Sausage	5	01/21/16	+	+
5.3	Sausage	5	01/21/16	+	+
5.6	Sausage	5	01/21/16	+	+
5.7	Sausage	5	01/21/16	+	+
5.8	Sausage	5	01/21/16	+	+
5.9	Sausage	5	01/21/16	+	+
6.3	Sausage	6	01/25/16	+	+
6.10	Sausage	6	25/01/16	+	+
6.11	Sausage	6	25/01/16	+	+
6.12	Sausage	6	25/01/16	+	+
			N °(+)	18	10

Table S3. *Listeria* spp. antimicrobial susceptibility testing.

ATB	Penicilin	Status	Ampicilin	Status	Trimetop.-Sulfametox.	Status	Gentamicin	Status
Isolate	MIC (S: ≤ 2 ug/mL)	S/R	MIC (S: ≤ 2 ug/mL)	S/R	MIC (R: ≥ 4/76 ug/mL)	S/R	MIC (S: ≤ 4 ug/mL)	S/R
3.4	1 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
4.1	1 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
4.2	1 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
4.3	1 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
4.4	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
4.5	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
4.6	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
4.8	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
5.1	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
5.3	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
5.6	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
5.7	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
5.8	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
5.9	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
6.3	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
6.10	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
6.11	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
6.12	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
ATCC Scott A	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S
ATCC Staphy.	0,5 ug/mL	S	0,5 ug/mL	S	1/19 ug/mL	S	1 ug/mL	S

Table S4. Adhesion potential of obtained *Listeria* spp. isolates (n= 18).

Source	Specie / Serotype	Isolate	AdhesionPotential
Sausage	*L. innocua*	3.4	Moderate
Sausage	*L. innocua*	4.1	Moderate
Sausage	*L. innocua*	4.2	Moderate
Sausage	*L. innocua*	4.3	Moderate
Sausage	*L. innocua*	4.4	Moderate
Sausage	*L. innocua*	4.5	Moderate
Sausage	*L. innocua*	4.6	Moderate
Sausage	*L. innocua*	4.8	Moderate
Sausage	(1/2a or 3a)	5.1	Moderate
Sausage	(1/2a or 3a)	5.3	Moderate
Sausage	(1/2a or 3a)	5.6	Moderate
Sausage	(1/2a or 3a)	5.7	Moderate
Sausage	(1/2a or 3a)	5.8	Moderate
Sausage	(1/2a or 3a)	5.9	Moderate
Sausage	(1/2a or 3a)	6.3	Moderate
Sausage	(1/2a or 3a)	6.10	Moderate
Sausage	(4b, 4d or 4e)	6.11	Weak
Sausage	(4b, 4d or 4e)	6.12	Weak

CHAPTER 2. *Listeria monocytogenes* adhesion ability and sensitivity to stressing factors

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RESUMO

Listeria monocytogenes é um patógeno capaz de aderir a superfícies abióticas de diversos materiais e formar biofilmes, o que pode explicar sua persistência em ambientes de processamento de alimentos. Este estudo teve como objetivo avaliar a capacidade de adesão de *L. monocytogenes* e a interferência de fatores de estresse na capacidade de adesão de cepas selecionadas. Isolados de *L. monocytogenes* do ambiente de processamento de produtos cárneos foram submetidos a testes de potencial de adesão e CIM contra quatro desinfetantes. A capacidade de adesão dos isolados também foi testada considerando: diluições de desinfetantes, concentrações de NaCl e sais de cura, tempo de incubação e temperatura. Cada isolado foi classificado de acordo com sua capacidade de adesão como fraco (1), moderado (4) ou forte (1). Os quatro desinfetantes testados foram eficazes na eliminação de *L. monocytogenes*. Os seis isolados selecionados para testes de estresse, apresentaram maior capacidade de adesão a 37 °C, e os meios com 5% de NaCl e amônia quaternária (1: 1,024) foram ineficazes na inibição da adesão ao poliestireno em alguns casos. Os dados coletados permitiram a identificação do potencial de adesão de *L. monocytogenes*, da eficácia dos sanitizantes testados no controle da contaminação por este patógeno e a capacidade de adesão na presença de amônio quaternário (1:1024) e 5% de NaCl.

Palavras-chave: *Listeria monocytogenes*, adesão, sanitizantes, fatores de estresse

ABSTRACT

Listeria monocytogenes is a pathogen capable of adhering to many surfaces and forming biofilms, which may explain its persistence in food processing environments. This study aimed to evaluate *L. monocytogenes* adhesion capacity as well as the interference of stress factors in the adhesion capacity of selected strains. *L. monocytogenes* isolates from meat processing enviroment were subjected to adhesion potential and MIC tests against four disinfectants. The isolates adhesion ability was also tested considering: culture media, disinfectant dilutions, NaCl and cure salts concentration, incubation time andtemperature. Each isolate was classified according to its adhesion ability as weak (1 isolate), moderate (4) or strong (1). The four disinfectants tested were effective in *L. monocytogenes* elimination. The six selected isolates to stress factors tests, showed higher adhesion capability at 37 °C, andmedia with 5 % NaCl and quaternary ammonium (1:1,024) were ineffective in inhibiting polystyrene adhesion in some cases. Collected data allowed identification of adhesion potential by *L. monocytogenes*, the effectiveness of the tested sanitizers to control contamination by this pathogen and adhesion capacity in the presence of quaternary ammonium and 5% Nacl.

Keywords: *Listeria monocytogenes*, adhesion, sanitizers, stress factors

1 INTRODUCTION

The elimination of *L. monocytogenes* in industrial food environments is difficult because of its known resistance to antimicrobial agents and chemical substances, such as acids and alkalis (MØRETRØ; LANGSRUD, 2004). However, the adhesion capacity and consequent formation of biofilms are considered as the main *L. monocytogenes* persistence factors in food industries (DYKES; MOORHEAD, 2002; DYKES, 2003; TAKHISTOV; GEORGE, 2004). This pathogen has proven adhesion properties to surfaces of different types of materials, often used in utensils and equipment in these industries (GANDHI; CHIKINDAS, 2007; PALMER; FLINT; BROOKS, 2007).

The growth and survival of a micro-organism is dependent on its ability to overcome environmental hurdles encountered during the manufacturing and conservation process, such as temperature, pH, water activity, metal ions and nutrient availability. Most bacteria, including *L. monocytogenes*, are generally resistant to small changes in a specific environmental parameter, but severe or multiple changes stimulate complex stress responses that are generally directed to survival instead of growth (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b).

The development of biofilms can be stimulated by different environmental factors on bacterial cells. Stressing situations, such as low availability of nutrients, low temperatures and sanitizers, are considered the main factors (JEFFERSON, 2004). In this sense, studies that seek to clarify the physiology and interfering factors in the adhesion and biofilms formation are relevant for its adequate control.

Since *L. monocytogenes* is a pathogen capable of adhering to various surfaces and forming biofilms, which may explain its persistence in food processing environments, this work evaluated *L. monocytogenes* adhesion capacity and the interference of stress factors on adhesion capacity of selected strains (*L. monocytogenes* strains belonging to lineages I and II, also coming from the meat processing environment, characterized in parallel works by strong adhesion potential (one isolate) and persistence capacity in the processing environment for 3 years (two isolates)), were incorporated into this work and tested with 3 selected isolates from sausage samples. These isolates were submitted to adhesion potential and minimum inhibitory concentration tests against four disinfectants.

2 MATHERIAL AND METHODS

2.1 *Listeria monocytogenes* isolates

As a selection criterion for subsequent tests, a *L. monocytogenes* clone representative of each cluster obtained in the Chapter 1 PFGE characterization (isolates 5.8, 6.3, 6.11) was chosen. Additionally, the following isolates were included in the study: CLIST 441 (collected in 2010 from beef in natura, in Mato Grosso), named as L1-SL3-ST3-CT4447 (L-lineage, SL-sublineage, ST-sequencetype, CT-core genome MLST) in "core genome MLST" analysis, with known strong adhesion capacity. From the "core genome MLST" analysis of *L. monocytogenes* genomes recovered in Brazil, a CT (CT4420) containing more than one isolate were obtained, suggesting an epidemiological link between them (persistence of this clone over the years). These isolates are respectively 19 (collected in 2009 from meat handlers) and 508 (collected in 2012 from fresh beef) designated L2-SL9-ST9-CT4420, were recovered from a same meat processing plant in Minas Gerais (MG).

2.2 *Listeria monocytogenes* adhesion potential

According to the recommendations of Djordjevic *et al.*, (2002) and Larivière-Gauthier *et al.*, (2014), the six selected isolates were subjected to adhesion capacity tests. The conditions for performing and interpreting the adhesion potential tests were the same as those described in chapter 1.

2.3. Effect of stressing factors on *Listeria monocytogenes* adhesion

2.3.1 Stressing factors

Four sanitizers were evaluated for their efficiency in eliminating *L. monocytogenes*, and its interference in the adhesion capacity. Sanitizer 1 was a quaternary ammonium based desinfectant (Kalyclean S 370, Kalykim, Alvorada, RS, Brazil); Sanitizer 2 was a desinfectant composed by peracetic acid, hydrogen peroxide and glacial acetic acid (Kalyclean S 380); Sanitizer 3 was a biguanide polyhexamethylene hydrochloride based desinfectant (Kalyclean S 311); Sanitizer 4 was a hydrogen peroxide based desinfectant (Kalyclean S 353).

Considering the isolates origin and the salts use in the mixtures preparations for sausages and meat products, the interference of 3 concentrations of curing salt (Exato, São Paulo, SP, Brazil) (BHI + 5% Cure Salt; BHI + 7.5% Cure Salt; BHI + 10% Cure Salt) and 3 concentrations of NaCl (Vetec, Rio de Janeiro, RJ, Brazil) (BHI + 5% NaCl, BHI + 7.5%

NaCl, BHI + 10% NaCl) were tested in *L. monocytogenes* adhesion. These factors were tested at 4 ^{0}C (refrigeration temperature), 12 °C (meat processing environment temperature) and 37 °C (ambient temperature), as well as in four incubation periods (24, 48, 72 and 96 hours) (Table S1).

2.3.2 Minimum inhibitory concentration of the sanitizers against *Listeria monocytogenes*

Listeria monocytogenes isolates obtained from the processing environment were subjected to a test for determination of the minimum inhibitory concentration (MIC) of the sanitizers 1, 2, 3 and 4. Isolates were overnight cultured in Brain Heart Infusion (BHI, Oxoid) at 37 °C, diluted in NaCl 0.85% (w/v) at 1:100, and 100 µL aliquots were pour plated on BHI agar (Oxoid). MIC values were assessed in 2 independent experiments, being sanitizers two-fold diluted in sterile distilled water until 1:2^{13} and 10 µL aliquots of each dilution was spotted on surface of BHI agar (Oxoid) previously plated with the tested cultures (CLSI, 2008). The highest dilution that resulted in absence of bacterial growth was considered as the MIC for the tested isolate (CLSI, 2008).

2.3.3 Effect of stressing factors in the adhesion capacity by *Listeria monocytogenes*

To test for sanitizers effect in the adhesion induction by *L. monocytogenes*, a 20 µL aliquot of each *L. monocytogenes* overnight culture was transferred to wells of a polystyrene microtiter plate containing 130 µL of BHI (Oxoid), and 30 µL of selected dilutions of sanitizers 1, 2, 3 and 4 (Table 2) were added. The same 20 µL aliquot of each *L. monocytogenes* overnight culture was transferred to wells containing 160 µL of BHI (Oxoid) supplemented with three concentrations of cure salt and NaCl, followed by incubation at temperatures and time periods described previously (Table S1). Then, the adhesion capacity assay was carried out as described above. All tests were performed in triplicate in three independent repetitions and included the appropriate negative controls (wells without sanitizers and salts) and blanks on the plates. Absorbance measurements obtained for each isolate were compared with the negative control.

3 RESULTS

3.1 *Listeria monocytogenes* adhesion potential

The adhesion potential of the isolates obtained from different samples are shown in Table 1. One isolate (CLIST 441) from raw beef showed strong adhesion potential, while two isolates from sausage (5.8, 6.3), one from raw beef (508) and one from meat handlers (19) showed a moderate adhesion potential, one isolate (6.11) from sausage were characterized as possessing a weak adhesion potential.

3.2 Determination of stressing factors effects on adhesion of *Listeria monocytogenes*.

Table 2 shows the MIC values for sanitizers 1, 2, 3 and 4 considering the selected isolates. Sanitizer 1 has inhibition potential to a dilution of 1:1,024, while the inhibition potential of sanitizer 2 ranged from 1:8 to 1:32, the inhibition potential of sanitizer 3 ranged from 1:64 to 1:256, inhibition potential of sanitizer 4 ranged from 1:16 to 1:64. These results show the higher efficiency of sanitizer 1 when compared to the others sanitizers, based on the response by each selected isolate.

Figure 1 shows the optical density of the *L. monocytogenes* isolates subjected to stress factors and time/ temperature conditions and tested for adhesion ability. According to Figure 1, the comparison of the optical densities obtained in the treatments (salts and disinfectants) in relation to the controls (BHI), showed the adhesion capacity of the isolate 6.11 in the presence of the disinfectant 1 (1:1,024) in the three temperatures tested, being that at 4 °C the incubation time of 48 hours obtained higher optical density, and at 12 °C the adhesion potential did not vary among the four incubation periods tested, at 37 °C the highest optical density was observed in the incubation period of 72 h. The results also showed that strains CLIST 441 and 508 presented moderate adhesion potential in the presence of 5% NaCl, as well as in the presence of disinfectant 1 (1:1,024), at 37 ° C in the incubation period after 72 h.

4 DISCUSSION

Listeria monocytogenes is known by its ability in adhering different types of surfaces persist in the environment and contaminate end products as observed in this study (Table 1). Silva *et al.*, (2017) evaluated *L. monocytogones* isolates from serotype 1/2c or 3c obtained from utensils and equipments used in the meat processing environment, andobserved strains with different adhesion potentials (strong, moderate and weak). The same adhesion potentials were observed by Galvão *et al.*, (2012), who evaluated *L. monocytogenes* isolates from serotypes 1/2a and 4b obtained from meat products processing environment and cattle carcasses.

Many studies have shown the *L. monocytogenes* adhesion capacity to materials that often come into contact with food, such as polystyrene, glass and stainless steel (DI BONAVENTURA *et al.*, 2008; GALVÃO *et al.*, 2012; LARIVIÈRE-GAUTHIER *et al.*, 2014; SILVA *et al.*, 2017). The adhesion ability of a microorganism is influenced by the adhering surface (physical–chemical properties and characteristics), the environment (pH, temperature,organic residues), and the microorganism itself (hydrophobicity, motility, and exopolysaccharides production) (MOLTZ; MARTIN, 2005).

Different sanitizers are employed by the food industry to control biofilm formation and microbial contamination of end products. Chlorine, quaternary ammonium compounds, hydrogen peroxide and sodium hypochlorite are some of the chemical compounds commonly used for sanitizing food processing plants (NORWOOD; GILMOUR, 2000; PAN; BREIDT; KATHARIOU, 2006). In the United States, the most used compounds for sanitization are hypochlorous acid, chlorine, iodine, ozone, hydrogen peroxide, peroxyacetic acid, quaternary ammonium compounds and ammonia acids (CARANDINA, 2013). According to Gaulin *et al.*, (2011) the following active ingredients are allowed in sanitizers used on organic food contact surfaces and equipment: hydrogen peroxide, ozone, peracetic acid / peroxyacetic acid, phosphoric acid, potassium hydroxide, sodium hydroxide.

The four (1, 2, 3 and 4) studied sanitizers were active against *L. monocytogenes* (Table 2). Quaternary ammonium compounds (sanitizer 1) act by inactivating enzymes, denaturing proteins and breaking down the microorganisms' cell membranes. The disinfectant activity of peracetic acid (sanitizer 2) occurs by the release of active oxygen from SS (sulfhydryl) and SH (sulfhydryl) bonds, promoting an increase in cell wall permeability, cytoplasmic content and genetic material, interfering with microorganisms' chemical survival reactions. Chlorinated compounds (sanitizer 3) act through several action mechanisms such as: destruction of protein synthesis, oxidative decarboxylation of aminioacids to nitriles and aldehydes, reactions with nucleic acids, metabolic imbalance after destruction of essential enzymes, inhibition of oxygen absorption and others. The action mechanism of peroxigenics (sanitizer 4) is due to the oxidation of the microorganisms cellular organelles, as described for peracetic acid compounds (CARANDINA, 2013).The sanitization step is the application of a chemical or physical agent to eliminate pathogenic microorganisms and to reduce the number of spoilage microorganisms to that considered to be a safe level and quality (CAMPDEPADRÓS *et al.*, 2012).

According to Figure 1, the temperature of 4 °C seemed to be determinant for the low performance of the isolates in adhering the polystyrene in the control condition (BHI) as well as in the stressful conditions (NaCl, curing salts and disinfectants). In all tested isolates at this temperature the quaternary ammonium (disinfectant 1 1:1,024) showed inefficiency in the adhesion inhibition when compared to the control (BHI). However, the optical density values for this treatment remained below the range considered for poor adhesion potential (under optimum conditions - Table S2), except for the isolate 6.11 where the O.D. values in quaternary ammonium presence reached the range considered for moderate adhesion potential (under optimum conditions).

The temperature of 12 °C also negatively influenced the adhesion capacity to polystyrene (Figure 1): isolates 5.8, 6.3, 441 and 19 presented optical density values for the control treatment (BHI) within the range considered for poor adhesion potential (under optimum conditions), and the isolate 508 had a better performance in adhering polystyrene since it reached optical density values in the control treatment (BHI), within the range considered for moderate adhesion potential (under optimum conditions). The disinfectants and salts tested at this temperature were efficient in inhibiting the *L. monocytogenes* adhesion to polystyrene, except for disinfectant 1 (1:1,024), which when tested for isolate 5.8, had an O.D. value higher than the O.D. value in the control situation (BHI), at 96-hour period. Isolate 6.11 was also able to achieve optical density values within the range considered as moderate adhesion potential (under optimum conditions) when tested in the presence of quaternary ammonium (disinfectant 1, 1:1,024).

When tested at 37 °C, the selected isolates presented a better performance in adhering the polystyrene in the control situation, when compared to the other tested temperatures (Figure 1). Generally, the isolates reached O.D. values able to reach the ranges of moderate and strong adhesion capacity. The incubation period non-linearly influenced the adhesion potential of the isolates tested in the control treatment (BHI).

The salts and disinfectants tested at 37 °C, were able to interfere negatively in the *L. monocytogenes* adhesion, except for some cases. Despite having reached O.D. values below the values obtained in the control situation (BHI) in some cases, disinfectant 1 (1:1,024) presented inefficiency in inhibiting polystyrene adhesion in all tested isolates. Particularly, isolates CLIST 441 and 508 reached O.D. values at 72 h within the range considered for moderate adhesion, and in the 96 h incubation period, isolate 508 reached O.D. values within the range considered as strong adhesion potential. NaCl at 5% was also unable to inhibit adhesion of isolates CLIST 441 and 508, and the incubation periods of 72 h and 96 h once more representative for O.D. values, within the range considered for moderate potential adhesion.

L. monocytogenes adhesion to polystyrene under the stressing conditions tested was shown to be influenced by temperature and strain-dependent issues, the incubation period influenced adhesion in a non-linear manner, since the optical densities varied for each treatment according to the time passage non-uniformly. The isolates presented higher adhesion ability when cultivated at 37 °C than at lower temperatures. These results are in agreement to the vision that temperature is a determining factor for *L. monocytogenes* adhesion, this was also observed in a similar study (MOLTZ; MARTIN, 2005).

Di Bonaventura *et al.*, (2008) studied 44 *L. monocytogenes* strains and found greater adhesion capacity on temperature of 37 °C when compared to 4, 12 and 22° C. Although stressing conditions were supposed to be considered as stimulants for biofilm formation (JEFFERSON, 2004), *L. monocytogenes* presented higher adhesion capacity in nutrient-rich and plastic surfaces (STEPANOVIC, *et al.*, 2004). The association between greater adhesion capacity and persistence of *L. monocytogenes* in industrial environments was identified by Norwood *et al.*, (2000), emphasizing the importance of pathogen control within industries in order to avoid contamination in food.

Despite the effectiveness of sanitizers in the MIC test, some isolates presented adhesion capacity in the presence of quaternary ammonium (1:1,024). Silva *et al.*, (2017) evaluated the effect of sanitizers in the biofilm formation and elimination of *Listeria monocytogenes* from meat processing environment equipaments and utensils, and showed the quaternary ammonium resistance by *L. monocytogenes* in the two tested situations.

Studies have shown that biofilms can be resistant to chemical cleaning and decontamination; the exopolysaccharide matrix formed creates a structure that difficult the cleaning sanitizers penetration, enabling their activity in the inner layers (BRIDIER *et al.*, 2011; FERREIRA *et al.*, 2014). *L. monocytogenes* is able to withstand a range of environmental stresses and to persist in food-handling environments, and some studies have isolated persistent strains in these environments even after sanitization (SOUMET;

RAGIMBEAU; MARIS, 2005). In addition, some authors have reported a correlation between the increase in the disinfectants MICs that are used commercially and the environmental persistence of isolates when compared to sporadic isolates (HOLAH *et al.*, 2002; KASTBJERG; GRAM, 2009).

In a similar way to this work, a study that evaluate the adhesion abilities of *L. monocytogenes* obtained from bovine carcasses and beef processing facilities, Galvão *et al.*, (2012) obtained isolates that showed higher adhesion capability in media with NaCl at 5% and 7%, and incubation at 25 °C and 37 °C. Interestingly, NaCl concentrations exceeding 5% have been reported to inhibit *L. monocytogenes* adhesion (JENSEN *et al.*, 2007).

Caly *et al.*, (2009) studied the adhesion capability of seven strains of *Listeria monocytogenes* to polystyrene and stainless-steel surfaces after cultivation at various NaCl concentrations. The results showed that adhesion between 0% and 6% NaCl was not different, whereas adhesionat 11% NaCl was significantly lower. This discrepancy in adhesion was corre-lated with the down-regulation of flagella at 11% NaCl. Only high salinity levels, close to nongrowth conditions, repressedthe expression of flagella, and consequently, decreased the *L. monocytogenes* adhesion capability.

5 CONCLUSION

Collected data allowed identification of adhesion potential by *L. monocytogenes* and the effectiveness of the tested sanitizers to control contamination by this pathogen. *L. monocytogenes* adhesion to polystyrene under the stressing conditions tested was shown to be influenced by temperature and strain-dependent issues, the incubation period influenced adhesion in a non-linear manner. Despite the effectiveness of sanitizers in the MIC test, some isolates presented adhesion capacity in the presence of quaternary ammonium (1:1,024) and 5% NaCl.

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Bibliography

BRIDIER, A. *et al.* Resistance of bacterial biofilms to disinfectants: a review. Biofouling, v. 27, n. 9, p. 1017–1032, 2011.

CALY, D. *et al.* Sodium chloride affects *Listeria monocytogenes* adhesion to polystyrene and stainless steel by regulating flagella expression. Letters in Applied Microbiology, v. 49, n. 6, p. 751–756, 2009.

CAMPDEPADRÓS, M. *et al.* Effectiveness of two sanitation procedures for decreasing the microbial contamination levels (including *Listeria monocytogenes*) on food contact and non-food contact surfaces in a dessert-processing factory. Food Control, v. 23, n. 1, p. 26–31, 2012.

CARANDINA, D. C. F. Avaliação de biofilmes formados por isolados de *Listeria monocytogenes* provenientes de laticínios e perfil de resistência a agentes sanitizantes. . [S.l.]: Universidade de São Paulo. , 2013

CLSI, CaLSI. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard—third edition—document m27-a3. CLSI, Wayne, Pa, USA, 2008.

DI BONAVENTURA, G. *et al.* Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. Journal of Applied Microbiology, v. 104, n. 6, p. 1552–1561, 2008.

DJORDJEVIC, D.; WIEDMANN, M.; MCLANDSBOROUGH, L. A. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Applied and Environmental Microbiology, v. 68, n. 6, p. 2950–2958, 2002.

FERREIRA, V *et al. Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. Journal of Food Protection, v. 77, n. 1, p. 150–170, 2014.

GALVÃO, N. N. *et al.* PFGE characterisation and adhesion ability of *Listeria monocytogenes* isolates obtained from bovine carcasses and beef processing facilities. Meat Science, v. 92, n. 4, p. 635–643, 2012.

HOLAH, J. T. *et al.* Biocide use in the food industry and the disinfectant resistance of persistent strains of Listeria monocytogenes and *Escherichia coli*. *Journal of applied microbiology*, v. 92, n. s1, 2002.

JEFFERSON, K. K. What drives bacteria to produce a biofilm? FEMS Microbiology Letters, v. 236, n. 2, p. 163–173, 2004.

JENSEN, A. *et al.* Sodium chloride enhances adherence and aggregation and strain variation influences invasiveness of *Listeria monocytogenes* strains. Journal of Food Protection, v. 70, n. 3, p. 592–599, 2007.

KASTBJERG, V. G.; GRAM, L. Model systems allowing quantification of sensitivity to disinfectants and comparison of disinfectant susceptibility of persistent and presumed nonpersistent *Listeria monocytogenes*. Journal of Applied Microbiology, v. 106, n. 5, p. 1667–1681, 2009.

LARIVIÈRE-GAUTHIER, G. *et al.* Analysis of *Listeria monocytogenes* strain distribution in a pork slaughter and cutting plant in the province of Quebec. Journal of Food Protection, v. 77, n. 12, p. 2121–2128, 2014.

MOLTZ, A. G.; MARTIN, S. E. Formation of biofilms by *Listeria monocytogenes* under various growth conditions. *Journal of food protection*, v. 68, n. 1, p. 92–97, 2005.

NORWOOD, D. E.; GILMOUR, A. The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady state multispecies biofilm. Journal of Applied Microbiology, v. 88, n. 3, p. 512–520, 2000.

PAN, Y.; BREIDT, F.; KATHARIOU, S. Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. Applied Environmental Microbiology, v. 72, n. 12, p. 7711–7717, 2006.

PROCESS, Sanitizing; FOOD, F O R; SURFACES, Contact. Allowed Detergents and Sanitizers for Food Contact Surfaces and Equipment in Organic Operations OVERVIEW : A Typical Cleaning and Sanitizing Process for Food. p. 1–4, 2011.

SILVA, D. A. L. *et al. Listeria* spp. contamination in a butcher shop environment and Listeria monocytogenes adhesion ability and sensitivity to food-contact surface sanitizers. Journal of Food Safety, v. 37, n. 2, p. e12313, 2017.

SOUMET, C.; RAGIMBEAU, C.; MARIS, P. Screening of benzalkonium chloride resistance in Listeria monocytogenes strains isolated during cold smoked fish production. Letters in Applied Microbiology, v. 41, n. 3, p. 291–296, 2005.

STEPANOVIĆ, S. *et al.* Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Letters in Applied Microbiology, v. 38, n. 5, p. 428–432, 2004.

THÉVENOT, D.; DERNBURG, A.; VERNOZY-ROZAND, C. An updated review of *Listeria monocytogenes* in the pork meat industry and its products. Journal of Applied Microbiology, v. 101, n. 1, p. 7–17, 2006.

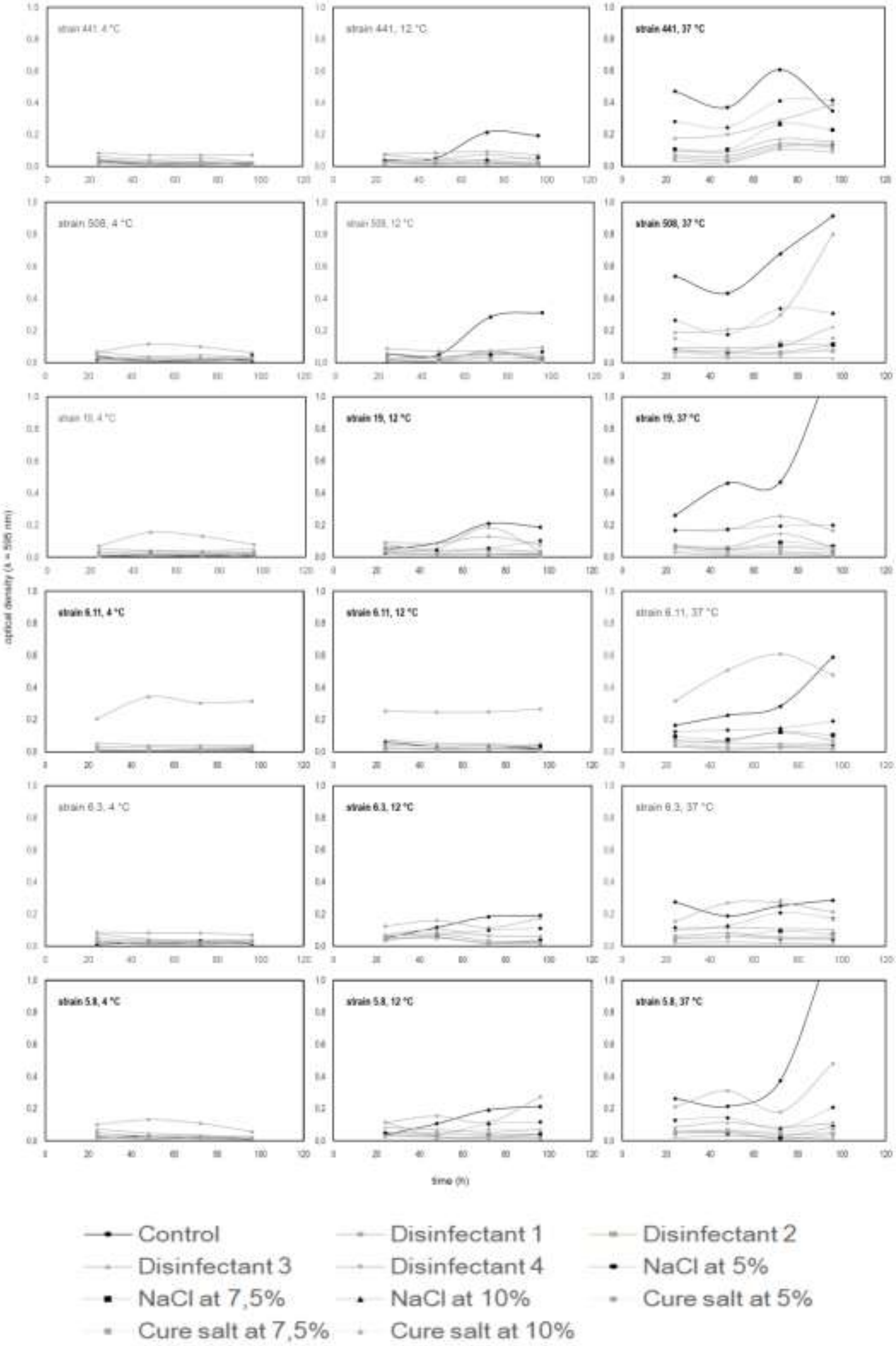
Table 1. Adhesion potential for *Listeria* spp. isolates from different sources.

Source	Specie / Serotype	Isolate	Adhesion Potential
Sausage	(1/2a or 3a)	5.8	Moderate
Sausage	(1/2a or 3a)	6.3	Moderate
Sausage	(4b, 4d or 4e)	6.11	Weak
Raw beef	(1/2b)	441	Strong
Meat Handlers	(1/2c)	19	Moderate
Raw beef	(1/2c)	508	Moderate

Table 2. Minimum Inhibitory Concentrations (MIC) of sanitizers 1 (Kalyclean S 370), 2 (Kalyclean S 380), 3 (Kalyclean S 311) and 4 (Kalyclean S 353) on growth of selected *L. monocytogenes* isolates.

Disinfectant	1 S370 (Quaternary Ammonium)	2 S380 (Peracetic acid/ Hydrogen peroxide/Glacial acetic acid)	3 S311 (Biguanide polyhexamethylene hydrochloride)	4 S353 (Hydrogen peroxide)
5.8	1:1024	1:32	1:256	1:32
6.3	1:1024	1:8	1:64	1:16
6.11	1:1024	1:32	1:256	1:32
19	1:1024	1:32	1:256	1:32
441	1:1024	1:32	1:128	1:64
508	1:1024	1:32	1:128	1:64
Scott A	1:1024	1:32	1:256	1:32
*Selected concentration for adhesion tests	1:1024	1:8	1:64	1:16

Figure 1. Absorbance values recorded from *L. monocytogenes* isolates subjected to adhesion potential test in microtitre plates, co-inoculated with four sanitizers at selected diluitions, NaCl and cure salts concentrations, submitted to three temperatures during four incubation periods.



SUPPLEMENTARY MATERIAL

Table S1. View of variables used to evaluate 6 *L. moncytogenes* isolates adhesion to polystyrene.

Incubation Time	24 h			48 h			72 h			96 h		
Incubation Temperature	4 °C	12 °C	37 °C	4 °C	12 °C	37 °C	4 °C	12 °C	37 °C	4 °C	12 °C	37 °C
Brain Heart Infusion (BHI) (C-)	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*
BHI + 5% Cure Salt	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*
BHI + 7.5% Cure Salt	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*
BHI + 10% Cure Salt	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*
BHI + 5% NaCl	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*
BHI + 7.5% NaCl	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*
BHI + 10% NaCl	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*
BHI + Disinfectant 1 (1:1024)	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*
BHI + Disinfectant 2 (1:8)	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*
BHI + Disinfectant 3 (1:64)	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*
BHI + Disinfectant 4 (1:16)	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*	6 *L. m*

6 *L. m* = six selected *L. monocytogenes* isolates tested.

Table S2. O.D. values of six selected *L. monocytogenes* isolates in the adhesion potential test.

37 °C / 24h O.D. λ= 595 nm	Ausent O.D.< or = 0,11	Weak 0,11 < O.D. <0,23	Moderate 0,23 < O.D. < 0,47	Strong O.D. > 0,47
5.8	-	-	0,24	-
6.3	-	-	0,27	-
6.11	-	0,12		-
441	-	-	-	0,5
19	-	-	0,45	-
508	-	-	0,41	-

CHAPTER 3. Biofilm and stress related genes screening by qPCR of *L. monocytogenes* in stainless steel biofilm systems under stress conditions.

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RESUMO

L. monocytogenes é reconhecida por sua capacidade de colonizar o ambiente e os equipamentos das indústrias de processamento de alimentos bem como persistir neste ecossistema por muitos anos. Essa persistência é mediada por múltiplos atributos deste patógeno, incluindo sua capacidade de formar biofilmes, replicar em baixas temperaturas e tolerar desinfetantes. O objetivo deste trabalho foi avaliar a capacidade de formação de biofilme em aço inoxidável por isolados de *L. monocytogenes* provenientes do ambiente de processamento de carne sob condições estressantes, bem como realizar uma análise preditiva da expressão dos genes relacionados à formação de biofilme e resistência ao meio ambiente por ensaios de qPCR. A formação de biofilme de *L. monocytogenes* em aço inoxidável foi testada em dois sistemas diferentes (microplaca e cupons) a 37 °C por 72 horas. As condições de estresse para cada linhagem testada (linhagem I e II) foram respectivamente de sal de cura 7.5% e amônia quaternária (1: 1,024). Os ensaios foram realizados como replicatas biológicas e os resultados foram expressos como unidades formadoras de colônia (UFC) por cm^2. A fim de obter uma análise preditiva da expressão dos genes relacionados à formação de biofilme e resistência ao estresse ambiental, os ensaios de qPCR foram realizados a partir das suspensões de biofilme obtidas a partir de testes em cupons de aço inoxidável. Os resultados mostraram que os isolados de ambas as linhagens testadas foram capazes de formar biofilme em aço inoxidável. Embora os tratamentos utilizados em cada linhagem tenham sido significativos ($p < 0,05$) na redução do biofilme formado, os isolados foram capazes de formar biofilme na condição de estresse avaliada. As suspensões de biofilme de *L. monocytogenes* a partir de testes em cupons de aço inoxidável deram resultados positivos em ensaios de qPCR para onze genes alvo testados. Os isolados testados foram capazes de formar biofilme em aço inoxidável na presença de agentes usualmente utilizados para preparações de embutidos e também nos procedimentos de limpeza e desinfecção da planta de processamento. A analise *in silico* do cg MLST destes isolados permitiu a identificação de regiões genômicas relacionadas à formação de biofilme e adaptação a condições ambientais estrassantes, em isolados das linhagens I e II, sendo ainda possível detectar a ação desta maquinaria genética na formação de biofilme em aço inoxidável sob ação de agentes estressantes, pela predição da expressão de onze gene alvo realizada por qPCR.

Palavras-chave: *Listeria monocytogenes*, biofilme, aço inoxidável, fatores de estresse

ABSTRACT

Listeria monocytogenes is notorious for its capacity to colonize the environment and equipment of food processing industries and to persist in the processing plant ecosystem, sometimes for many years. Such persistence is mediated by multiple attributes of this pathogen, including the pathogen's capacity to form biofilms, replicate at low temperatures and tolerate disinfectants.The objective of this study was to evaluate the ability of biofilm formation in stainless steel by *L. monocytogenes* isolates from the meat processing environment under stressful conditions, as well as to perform an expression predictive analysis of the genes related to biofilm formation and resistance to environmental stress by qPCR assays. *L. monocytogenes* biofilm formation on stainless steel was tested in two different systems (microplate and coupons) at 37 °C for 72 hours.The stress conditions for each lineage tested (lineage I and II) were respectively 7.5% cure salt and quaternary ammonium compound (1: 1,024). The assays were performed as biological triplicates and results were expressed as colony forming units (CFU) per cm^2. In order to obtain an expression predictive analysis of the genes related to biofilm formation and resistance to environmental stress, qPCR assays were performed from the biofilm suspensions obtained from stainless steel coupons tests.The results showed that the isolates of both lineages tested were able to form biofilm in stainless steel. Although the treatments used in each lineage were significant ($p < 0.05$) in the reduction of the formed biofilm, the isolates were able to form biofilm in the evaluated stress condition. *L. monocytogenes* biofilm suspensions from stainlees steel coupons tests, gave positive results in qPCR assays for eleven target genes tested. The tested isolates were able to form stainless steel biofilm in the presence of agents usually used for sausage preparations and also in the processing plant cleaning / disinfection procedures. The *in silico* analysis of cg MLST allowed the identification of genomic regions related to biofilm formation and adaptation to stressful environmental conditions in lineages I and II isolates. It is also possible to detect the action of this genetic machinery in the stainless steel biofilm formation under action of stress factors, by predicting the expression of eleven target genes performed by qPCR.

Keywords: *Listeria monocytogenes*, biofilm, stainless steel, stress factors

1 INTRODUCTION

Listeria monocytogenes is notorious by its capacity to colonize the environment and equipment of food processing industries and to persist in the processing plant ecosystem, sometimes for decades (AZIZOGLU *et al.*, 2015). Such persistence is mediated by multiple attributes, allowing this pathogen to adhere and to form biofilms on several materials, such as stainless steel, glass and polymers (PIETA *et al.*, 2014). Also, *L. monocytogenes* can grow in the cold, tolerate the presence of disinfectants, and resist to attack by phages (AZIZOGLU *et al.*, 2015). However, little is known about the molecular mechanisms responsible for these processes (PIETA *et al.*, 2014).

Biofilm formation is of pivotal importance: not only are bacteria in the sessile biofilm community is more difficult for removal than loosely adherent cells, but also the existence of bacteria in a biofilm impacts adaptive attributes, potentially resulting in enhanced tolerance towards various stresses. Indeed, extensive evidence indicates that bacteria in biofilms from diverse ecosystems exhibit enhanced tolerance to disinfectant and other antimicrobial agents (AZIZOGLU *et al.*, 2015). Several environmental factors that are commonly related to food and food processing plants, such as concentration of salt and sugar, pH, temperature and the presence of nutrients, are important in the initial adhesion and subsequent biofilm formation by *L. monocytogenes* (PIETA *et al.*, 2014). *L. monocytogenes* has evolved over a long period of time to acquire a diverse set of genes and corresponding proteins, each with its own unique properties and functions in the pathogenicity and survival of this microorganism under stress conditions (PIETA *et al.*, 2014).

A previous study identified *Listeria monocytogenes* long-term persistence in a Brazilian beef prossessing plant, based on core genome multilocus sequence typing (cgMLST) and single nucleotide polymorphisms analysis (wgSNP) (Camargo *et al.*, 2019). In the same study, it was detected another strain carring a PMSC (premature stop codon) in *agrC*, curiously, a previous screening in our lab showed that this strain had a strong adhesion on polystyrene microtiter plates (24 h incubation) and stainless steel coupons (48 h). In this way, the objective of this study was to evaluate the ability of biofilm formation in stainless steel by *L. monocytogenes* isolates from the meat processing environment under stressful conditions, as well as to perform an expression predictive analysis of the genes related to biofilm formation and resistance to environmental stress.

2. MATERIAL AND METHODS

2.1 Study design

Considering these findings related to *L. monocytogenes* strains from our lab collection, and based on genomics characteristics (Table 1), such as presence of genes (conserved or not) related to biofilm formation and stress resistance (Table S1), four isolates associated to food production environment were subjected to phenotypic assays aming to test their adhesion ability under different stressful conditions on stainless steel wells. In a subsequent step, the biofilms were grown on under stressful conditions on stainless steel coupons, where the viable sessile cells were enumerated, and the expression of genes related to biofilm formation and stress survival were predicted.

2.2 Screening of *L. monocytogenes* adhesion in stainless steel under stressing conditions (NaCl, Cure salts, QAC)

From the results obtained in Chapter 2, isolates CLIST 441 and 508 (and their comparative controls - respective isolates 07 and 19) were selected for stainless steel adhesion capacity tests in the presence of cure salt and NaCl concentrations as well as quaternary ammonium (disinfectant 1 1: 1,024), considering the incubation time of 72 h and 37 °C temperature.

To test sanitizers effect in the adhesion induction by *L. monocytogenes*, a 20 μL (10^9 cell/ml) aliquot of each *L. monocytogenes* culture was transferred to wells of a stainless steel microtiter plate containing 130 μL of BHI (Oxoid), and 30 μL of sanitizer 1 (1:1,024) were added. The same 20 μL aliquot of each *L. monocytogenes* culture was transferred to wells containing 160 μL of BHI (Oxoid) supplemented with three concentrations of cure salt (Exato, São Paulo, SP, Brazil) (BHI + 5% Cure Salt; BHI + 7.5% Cure Salt; BHI + 10% Cure Salt) and 3 concentrations of NaCl (Vetec, Rio de Janeiro, RJ, Brazil) (BHI + 5% NaCl, BHI + 7.5% NaCl, BHI + 10% Cure Salt), followed by incubation for 72 hours at 37 °C. After incubation, the cultures were discarded, and the wells were washed with PBS (pH 7.2) three times to remove nonadhered cells. The cells were fixed by addition of methanol (Sigma-Aldrich), and air-dried for 10 min. Then, crystal violet solution (1% w/v) was added, and after 15 min the plates were washed with water. After drying, 95% ethanol was added to each well (incubation for 30 min at room temperature), the contents of the wells were transferred to a polystyrene microplate and the absorbance was measured (λ = 595 nm, Multi Scan FC, Thermo Fisher Scientific, Walthan, EUA). All tests were performed in six independent replicates and included the appropriate negative controls (wells without sanitizers and salts) and blanks on the plates. Absorbance measurements obtained for each isolate were compared with the negative control. Since this is a screening step to select conditions to be used in thenext tests, the observations were based only on the comparison of numerical data.

2.3 Phenotypical characterization of adhesion features of *Listeria monocytogens* under specific stressing conditions

2.3.1 *L. monocytogenes* biofilm formation on stainless steel microplate

Considering additional data results lineage I isolates (441 and 7) were tested for biofilm formation in stainless steel in the presence of 7.5% curing salt (Exato, São Paulo, SP, Brazil) (37 °C / 72 h); the isolates of lineage II (508 and 19) were tested for stainless steel biofilm formation in the presence of quaternary ammonium (disinfectant 1 1: 1,024) (Kalyclean S 370, Kalykim, Alvorada, RS, Brazil) (37 °C / 72 h).

To test cure salt effect in the lineage I isolates biofilm formation instainless steel, a 20 µL (10^9 cell/mL) aliquot of each *L. monocytogenes* isolate was transferred to wells of a stainless steel microtiter plate containing 160 µL of BHI (Oxoid) supplemented with cure salt at 7.5%. The same 20 µL (10^9 cell/mL) aliquot of each lineage II isolate was transferred to wells of a stainless steel microtiter plate containing 130 µL of BHI (Oxoid), and 30 µL of sanitizer 1 (1:1,024) were added. The biofilm system was incubated for 72 hours at 37 °C, under constant agitation at 15 rpm. After incubation, the cultures were discarded, and the wells were washed with PBS (pH 7.2) three times to remove non adhered cells. Remaining adhered cells were removed by ten vigorous pipettings in circular motions with 200 uL 1% buffered peptone saline solution added 1% tween 80 (Dinâmica, Indaiatuba, SP, Brazil). To estimate *L. monocytogenes* adhesion, tenfold dilutions of the biofilm suspensions were done in PBS, drop plated (10 µL) on BHI agar andincubated at 37 °C for 24 h, according to Herigstad *et al.*, (2001) with modifications. The assays were performed as biological triplicates and included the appropriate controls (wells without sanitizer and salts)to check for significant differences by analysis of variance ($p < 0.05$) using the program XLSat 2010.2.03 (Addinsoft, New York, NY, USA).The results were expressed as colony forming units (CFU) per cm^2, for sessile cells (0.2 mL of the homogenate corresponds to 1.6 cm^2).

2.3.2 *L. monocytogenes* biofilm formation on stainless steel coupons

L. monocytogenes biofilms were grown on stainless steel (AISI 304, 3.8 cm^2) coupons. Prior to inoculation, the coupons were cleaned and prepared according to Winkelströter *et al.*, (2011) placed in 24 wells polystyrene microtiter plates (TPP, Switzerland) with the following contents: to lineage I, a 250 µL (10^9 cell/mL) aliquot of each *L. monocytogenes* isolate was transferred to wells containing 1.75 mL of BHI (Oxoid) supplemented with cure salt at 7.5%. The same 250 µL (10^9 cell/mL) aliquot of each lineage II isolate was transferred to wells containing 1.42 mL of BHI (Oxoid), and 328 µL of sanitizer 1 (1:1,024) were added. The biofilm system was incubated for 72 h at 37 °C, under constant agitation at 15 rpm. The assays included the appropriate controls (wells without sanitizer and salts).To enumerate *L. monocytogenes* cells attached to the coupons, each coupon was rinsed three times with PBS to eliminate non-adhered cells, transferred to test tubes containing 2 mL of 1% buffered peptone saline solution added 1% tween 80, treated for 2 min in ultrasound bath (50–60 kHz) and vortexed for 1 min according to Leriche *et al.*, (1995) with modification. Ten-fold dilutions of the biofilm suspensions were done in PBS, drop plated (10 µL) on BHI agar andincubated at 37 °C for 24 h, according to Herigstad *et al.*, (2001). The assays were performed as biological triplicates and included the appropriate controls (wells without sanitizer and salts) to check for significant differences by analysis of variance ($p < 0.05$) using the program XLStat 2010.2.03 (Addinsoft, New York, NY, USA). Results were expressed as colony forming units (CFU) per cm^2, for sessile cells (2 mL of the homogenate corresponds to 3.8 cm^2). In this study, to consider that a biofilm was formed, at least 10^3 adhered cells per cm^2 should be quantified (MINEI *et al.*, 2008; WINKELSTRÖTER; DE MARTINIS, 2015).

2.4 Molecular characterization of adhesion features of *Listeria monocytogenes* under specific stressing conditions

2.4.1 Panel of selected genes

In order to obtain an expression predictive analysis of the genes related to biofilm formation and resistance to environmental stress, qPCR assays were performed from the biofilm suspensions obtained from stainless steel coupons tests, led to RNA production detection related to the eleven selected genes (Table 2).

2.4.2 Gene expression prediction by qPCR

Two independent biological repetitions of *L. monocytogenes* biofilm suspensions from stainless steel coupons tests were centrifuged at (5,000 rpm / 10 min). The supernatant was discarded and the pellet resuspended in 100 µL of lysozyme. After vortexing and incubation (37 °C / 5 min), 350 µL of the Buffer RLT and 3.5 µL of β-mercaptoethanol were added, after vortexing followed by centrifugation at (13,000 rpm / 2 min), the supernatant was transferred to a new tube and resuspended in 250 µL of ethanol (96%). RNA extraction was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, USA) and converted to cDNA by the GO Script Reverse Transcription System (Promega) according to the manufacturer's instructions.

The primers used in this study were obtained from Life Technologies Brasil (São Paulo, Brazil) and they detected *L. monocytogenes*. Primers sequences were shown in Table 2. The amplification was performed using a real-time PCR system Rotor-Gene Q (Qiagen, Hilden, Germany) equipped with the Rotor-Gene Q Series Software (Qiagen, Hilden, Germany) for data acquisition and analysis of results. All PCR amplifications were performed in 20 µL reaction volume with 2 µL of suspension lysate (target cDNA), 10 µL of GoTaq qPCR Master Mix (Promega, Madison, USA), 0,4 µL of each primer solution (0.20 mM of each primer) and 7.2 µL of purified water. The conditions for PCR reaction comprised an initial denaturation step at 95 °C for 2 min and 45 cycles of 95 °C for 15 s and 60 °C for 60 s. The thermocycling program was followed by an additional heating step done at 1 °C/s, from 60 °C to 95 °C, to obtain the melting curve. Specificity of amplification was confirmed by agarose gel electrophoresis and the results were evaluated according to the positive (*L. monocytogenes* isolates DNA) and negative (purified water) controls.

3 RESULTS AND DISCUSSION

3.1 Screening of *L. monocytogenes* adhesion in stainless steel under stressing conditions (NaCl, Cure salts, QAC)

Figure 1 shows absorbance values recorded from *L. monocytogenes* isolates subjected to adhesion potential test in stainless steel microtitre plate, co-inoculated with, disinfetant 1 (1:1,024), NaCl and cure salts concentrations, submitted to incubation for 72 h at 37 °C. According to the results, the comparison of the optical density values among lineage I isolates showed a better steel adhesion capacity of isolate CLIST 441 than to isolate 7. For this last isolate, results showed values of optical density in the presence of the disinfectant 1 (1: 1024) slightly higher than the values obtained in the control (BHI).

The treatments of 5% NaCl, 7.5% NaCl, 10% NaCl and 5% C.S. negatively influenced the adhesion capacity of the isolate CLIST 441 to the steel, the O.D. values obtained in the respective treatments were inferior to the control (BHI) O.D. values. The treatments 7.5% C.S., 10% C.S. and quaternary ammonium (1: 1,024) were stimulants for the adhesion capacity of the isolate CLIST 441 to steel, and the cure salt at 7.5% had a remarkable performance in the adhesion potential of this isolate to steel, the optical density value for these treatments was three times higher than the control O.D. value.

Among isolates of lineage II, the comparison of optical density values revealed greater adhesion ability to the steel by isolate 19, when compared to isolate 508. All the tested treatments influenced negatively the adhesion capacity to the steel of isolate 19 when compared to the control (BHI). For the isolate 508, the optical density values obtained in the 5% NaCl, 7.5% NaCl, 10% NaCl, 5% C.S. and 10% C.S. treatments were below to the control O.D. value, indicating a negative influence of each treatment on the isolate 508 steel adhesion ability. The opposite occurred for the 7.5% C.S. treatment, which reached a value close to that obtained for the control (BHI), and for quaternary ammonium (1: 1,024), whose O.D. value was higher than the control.

In accordance with the results in chapter two (taking into account the change of adhesion surface), isolates CLIST 441 and 508 showed adhesion to steel in the presence of salts and quaternary ammonium (1: 1,024). Considering a better performance in adhering the steel of isolate CLIST 441 in the presence of curing salt 7.5% and isolate 508 in the presence of quaternary ammonium (1: 1,024), added to the physical limitation of the stainless steel systems to be used in the following tests (chapter 3), conditions were determined: lineage I isolates (CLIST441 and 7) will be tested for biofilm formation in stainless steel in the presence of 7.5% curing salt (72 h / 37 °C); the isolates of lineage II (508 and 19) will be tested for stainless steel biofilm formation in the presence of quaternary ammonium (1: 1024) (72h / 37 °C).

3.2 Stainless steel biofilm formation under stress conditions

Stainless steel is widely used in food industry plants and facilities because it exhibits characteristics such as low and high temperature resistance, corrosion resistance, low ion migration, smooth surface and low porosity, which hinders the adhesion and retention of microorganisms (CABEÇA, 2006). The neutralization in relation to food, preventing the organoleptic properties of food from being altered (STEINER; MARAGOS; BRADLEY JR, 2000). In contrast, the stainless steel surfaces used in plants and equipment for handling, storage or processing food are recognized as the main sources of microbial contamination (CHMIELEWSKI; FRANK, 2003).

In this work, both lineage 1 and 2 isolates showed a high capacity in forming biofilms in the two stainless steel systems tested at 37 °C / 72 h (Figures 2 and 3). Biofilm formation was considered when the density of adhered cells was at least 10^3 CFU/cm² (MINEI *et al.*, 2008; WINKELSTRÖTER; DE MARTINIS, 2015). Figure 2 shows that lineage I isolates, CLIST 441 and 7 respectively presented counts in the control situation (BHI), that reached 7.64 log CFU/cm² and 6.65 CFU/cm² in the stainless steel microplate system, and for the stainless steel coupon system the counts were respectively, 5.8 CFU/cm² and 4.71 CFU/cm². Isolate CLIST 441 presented better performance in forming biofilm when compared to isolate 7, a result that is in agreement with the strong adhesion potential demonstrated in previous tests (data not shown). According Figure 3, isolates from lineage II, 508 and 19 respectively reached counts of 7.45 log CFU /cm² and 7.11 CFU/cm² in the steel microplate system, and for the stainless steel coupon system the counts were respectively, 6.51 CFU/cm² and 6 CFU/cm² in the control situation (BHI). The results also showed that in both tested *L. monocytogenes* lineages, the system used to evaluate the biofilm cell viability seemed to influence the performance of each strain, since higher CFU/cm² counts were obtained in the microplate system when compared to the coupons system. The inoculum concentrations and amount of culture medium used should be considered proportional for each system, but the cell detachment method varied between the tests.

In agreement to this study, Carandina *et al.*, (2013) in an evaluation of biofilms formed by isolates of *L. monocytogenes* from dairy products, it was possible to verify the biofilm formation on the stainless steel surface after 72 h of incubation, at 35 °C in BHI broth. In the present study, in both stainless steel tested systems, the plate count method revealed that 7.5% curing salt treatment (composed of 86% non-iodized salt, 11% nitrite and 3% sodium nitrate) used in the biofilm formation of the lineage I isolates, was efficient in reducing the *L. monocytogenes* population adhered to steel when compared to the results for the biofilm cultured in untreated (control) BHI broth ($p < 0.05$) (Figure 2). For the isolate CLIST 441, the microplate count decreased to 5.7 CFU/cm² (control count: 7.64 CFU/cm², p

<0.0001), and in the coupon system reduced to 3.46 CFU/cm² (control count: 5.8 CFU/cm², p = 0.003) when compared to the controls. The microplate count of the biofilm formed by the isolate 7 in the presence of salt cure reduced to 5.56 CFU/cm² (control count: 6.65 CFU/cm², p = 0.001), and in the coupon system the plating count reduced to 3.5 CFU/cm² (control count: 4.71 CFU/cm², p = 0.017) when compared to the control counts.

Despite the treatment used was significant for the biofilm reduction by isolates from lineage 1, it was possible to demonstrated the ability by these isolates to adhere to the steel and to form biofilm in the presence of compounds commonly used in meat mixtures preparations and sausages, in order to inhibit the microbial multiplication primarily through osmotic stress. Since the tested treatment is usually used against microorganisms in the form of free cells, this approach was based on the ability of the isolates to adhere to the steel in the presence of 7.5% cure salt in additional tests and in the possibility of evaluation the effect of these compounds on *L. monocytogenes* in the condition of sessile cells.

L. monocytogenes is able to multiply at high salt concentrations, being salt tolerance related to pH and temperature conditions (THÉVENOT; DERNBURG; VERNOZY-ROZAND, 2006b). The unusual application of curing salts in biofilm formation tests contributes to lack of comparative data in related studies. An experimental design study was used to assess quantitatively the effects and interactions of temperature, $NaNO_2$ (sodium nitrite), and others factors on the growth kinetics of *L. monocytogenes* (BUCHANAN; PHILLIPS, 1990) and results showed that the primary inhibitory action against *L. monocytogenes* involves a bacteriostasis, resulting in an extension of the lag phase and a growth depression rate. Farber *et al*., (1991) indicated that nitrite accelerated the inactivation of *L. monocytogenes* in fermented meats. Interestingly, Mackey *et al.*, (1990) observed that the addition of curing salts alone produced a marked increase in heat resistance of *L. monocytogenes* in cured products.

Lineage II plating count results revealed that quaternary ammonium (1:1,024) was efficient in the reduction of biofilm production by selected isolates in both stainless steel system evaluated ($p < 0.05$) (Figure 3). Microplate count of isolate 508 decreased to 4.43 CFU/cm² (control count: 7.45 CFU/cm², $p < 0.0001$), and in the coupon system reduced to 2.75 CFU/cm² (control count: 6.51 CFU/cm², $p < 0.0001$) when compared to the controls. The microplate count of the biofilm formed by the isolate 19 in the presence of QAC's (1:1,024) reduced to 5.46 CFU/cm² (control count: 7.11 CFU/cm², p = 0.006), and the plating count in steel coupon system reduced to 2.38 CFU/cm² (control count: 6.01 CFU/cm², $p < 0.0001$) when compared to the counts in the control.

These results are in agreement with the assertion that on average the sanitizers reduce from 2 to 4 log CFU/cm² of *L. monocytogenes* in biofilm, although the biofilm removal depends on factors such as biofilm stage, surface and the substrate in which the biofilm was produced (NORWOOD; GILMOUR, 2000; PAN; BREIDT; KATHARIOU, 2006; YANG, HUA *et al.*, 2009). The quaternary ammonium based disinfectant was efficient in the reduction of biofilm formed by the lineage II ($p < 0.05$), but the tested isolates presented biofilm formation capacity in the microplate system in the presence of this compound, and despite the counts by the plate count method, in the stainless steel coupons system did not reach 3 log CFU/cm^2 it was possible to observe the *L. monocytogenes* adhesion to the steel coupons in the presence of QAC (1: 1,024).

Quaternary ammonium compounds are disinfectant cationic surfactants, effective against Gram positive bacteria. They are indicated in the disinfection of open equipment, parts, utensils and surfaces of refrigerators, abattoirs, dairy products and food industries (CABEÇA, 2006). They have the characteristics of being non-corrosive, with low toxicity, not irritating the skin of manipulators, and their activity is not affected by organic residues (CHMIELEWSKI; FRANK, 2003). Resistance against sanitizing agents of *L. monocytogenes* in biofilms was demonstrated by (CABEÇA; PIZZOLITTO; PIZZOLITTO, 2012); the reduction of cell viability of *L. monocytogenes* adhered to stainless steel coupons in the presence of quaternary ammonium compounds was also observed, biofilm formantion reduced to 1.4 log CFU/cm^2 when compared with positive control (6.2 log CFU/cm^2).

Isolates 19 (collected in 2009 from meat handlers) and 508 (collected in 2012 from fresh beef) designated L2-SL9-ST9-CT4420 were recovered from the same meat processing plant in Minas Gerais (MG); this clone have persisted in this plant over the years, besides this ability to persist in the processing line. Based on the obtained results, it was possible to characterize the clone's ability to adhere stainless steel surfaces and form biofilm as well as to resist the action of quaternary ammonium compounds (1: 1,024), which may explain the contamination of final products by cross-contamination (Figure 3).

In the same way, Poimenidou *et al.*, (2016) investigated the correlation between biofilm forming ability, tolerance to disinfectants of *L. monocytogenes* strains. The results showed that, the MIC QAC's was positively correlated with the biofilm-forming ability on stainless steel (P = 0.03). Curiously, among all strains, the greatest biofilm producer was a persistent strain with significant tolerance to QAC's.

3.3 qPCR analyses

L. monocytogenes biofilm suspensions from stainlees steel coupons tests gave positive results in qPCR assays. Table 3 shows the positivity panel of gene expression for each target primer screened. The cycle threshold values variation for each primer tested are shown in the Table 4. The amplification size determined by the agarose gel analysis, obtained from cDNA amplification of *L. monocytogenes* biofilm in stainless steel, was according to the works referenced for each selected primer (Table 4). The melt temperature (T_m) and cycle thereshold (CT) values for each gene searched, in relation to each control DNA tested are shown in Table 4.

According to Table 3, only it was not possible to predict the expression of the *flaA* and *lmo0446* genes in the biofilm suspensions of lineage 1 isolates, both in the control (BHI) situation and in the curing salts effect on biofilm induction tests. It was possible to predict the expression of all selected target genes in the biofilm suspensions of lineage 2 isolates. In particular, it was not possible to detect expression of the *ldE* gene in one of the repetitions of the isolate 508 under the effect of QAC's, and similarly, for the isolate 19, it was not possible to detect expression of the *agrC* gene in one of the biofilm formation repetitions in the presence of QAC's.The amplification success in molecular biology techniques depends to a great extent on the efficiency of the extraction protocols that should allow the recovery of high quality genetic material and quantity (LIU *et al.*, 2008; MAFRA *et al.*, 2008; PEANO *et al.*, 2004). A variety of inhibitory agents such as extraction reagents and food matrix may be present in the samples and influence the results obtained by real-time PCR (GUESCINI *et al.*, 2008; LIU *et al.*, 2008; PEANO *et al.*, 2004).

The mechanisms and mediators of the cell attachment have been a focus of biofilm research for a long time, and flagella are considered to play an important role on seed cell attachment in several bacteria (OUYANG *et al.*, 2012). In *L. monocytogenes*, several genes related to regulation of flagella synthesis, flagella structure (*flaA*) and motility have been linked to biofilm formation (OUYANG *et al.*, 2012). Studies on the role of *L. monocytogenes* flagella in attachment to abiotic surfaces suggests that, in the absence of motility, flagella have a role as adhesins in initial surface attachment to stainless steel (VATANYOOPAISARN *et al.*, 2000).

Lemon *et al.*, (2007) suggests that biofilm development by *L. monocytogenes* proceeds via both growth of initially surface-adhered cells and ongoing recruitment of motile cells from the planktonic phase. In thinking about how flagellum-mediated motility is critical for *L. monocytogenes* biofilm formation, a model was proposed, that the primary role of flagellum in surface-associated biofilm formation is to provide the force necessary to overcome repulsive forces that might exist between the bacteria and the surface (LEMON; HIGGINS; KOLTER, 2007). Incorporated in this idea is also the concept that the contribution of flagellum-mediated motility is simply to increase the probability of encountering a surface. It appears that during biofilm development in many other motile bacteria, motility and extracellular matrix production are inversely regulated, such that once motile cells contact a surface they switch to producing matrix (LEMON; HIGGINS; KOLTER, 2007). Studies that determine whether a similar situation exists in *L. monocytogenes* and how it can be regulated are necessary.

Besides flagella, the bacterial quorum sensing (QS) systems also play a role in the early stages of biofilm formation. In *L. monocytogenes*, the peptide-mediated QS system (Agr) is involved (OUYANG *et al.*, 2012). The accessory gene regulator (*agr*) locus encodes for a peptide sensing system that is found in many Gram positive organisms and has pleiotropic effects (ZETZMANN *et al.*, 2019). For *L. monocytogenes*, it has been shown that the *agr* system is enrolled in the regulation of virulence, biofilm formation and is required for environmental survival effects (ZETZMANN *et al.*, 2019).

The locus *agr* is composed of four genes, co-transcribed in an operon (agr BDCA), which encode four proteins. *agrB* is responsible for the expression of a membrane protein, whose function is to export the peptide expressed by *agrD*, which is transformed into a mature auto-inducing peptide (AIP). AgrC (histidine kinase) and AgrA (auto-regulatory) form a transduction signal of a two-component system. When the extracellular AIP concentration reaches a certain level, the system is activated by quorum sensing, through the detection of AgrC-AgrA, exerting regulatory effects on target substrates and inducing its own production (positive auto-regulation) (GANDRA *et al.*, 2019). The activated system regulates the genes encoding the adhesins required for biofilm formation. Studies showed that mutations of *agr* locus genes decrease the ability of *L. monocytogenes* to adhere to abiotic surfaces and to form biofilm, and may also affect the infective capacity (GANDRA *et al.*, 2019). In a study carried out by Pöntinen *et al.*, (2017), a deletion mutant in the AgrC sensor histidine kinase of the *agr* system displayed increased sensitivity to high concentrations of salt; it was hypothesized that a reduced resistance to osmotic stress may lead to increased lysis of bacteria and, consequently, reduced surface-attached biomass.

Bacterial genomes contain a large arsenal multi-drug transporter genes (MDR), many of which are highly similar and possess overlapping substrate specificity, with their physiological functions mainly related to efflux pumps (ZEEVI *et al.*, 2013). It is believed that MDR transporters evolved independently during evolution to deal with a wide range of physiological substrates, allowing bacteria to survive several ecological niches (ZEEVI *et al.*, 2013). Studies in the last decade have revealed several functions of MDR transporters that are not related to drug efflux, for example, involvement of MDR transporters in lipid transport, pH homeostasis, virulence, and quorum sensing has been documented, with fatty acids, ions, bile salts, antibacterial peptides, and precursors of quorum-sensing molecules suggested as natural substrates (ZEEVI *et al.*, 2013). MdrM and MdrT belong to the major facilitator superfamily (MFS) of MDR transporters and are closely related to the well characterized MDR transporter, QacA, of *Staphylococcus aureus*. MDR transporters, such as QacA, are notorious for their ability to confer resistance to a wide variety of toxic compounds and drugs, including antibiotics, by utilizing proton motive force to actively extrude these compounds outside the cell (ZEEVI *et al.*, 2013).

Despite the advance in understanding of MDR transporters, *in silico* prediction of MDR transporter functions remains challenging. The initial discovery that the *L. monocytogenes* MdrM transporter modulates the type I interferon response raised many questions regarding the mechanism of this function. Primarily, it was not clear whether this function evolved specifically to subvert the host immune system or, alternatively, represents the inadvertent consequence of a more basic bacterial physiological function. Later the report that c-di-AMP is secreted by MdrM and leads to IFN-β induction further highlighted the need to better understand the natural biological process that involves MdrM and c-di-AMP (ZEEVI *et al.*, 2013).

It was proposed that MdrM and MdrT transporters extrude cyclic-di-AMP (c-di-AMP) during *L. monocytogenes* intracellular growth, which in turn activates infected macrophages to elicit the IFN-β response. Indeed, c-di-AMP activates a robust type I interferon response when added exogenously; however, a physiological association between c-di-AMP and the MDR transporters was not established. Notably, several reports had indicated that c-di-AMP serves as a second messenger molecule that influences central cellular processes of bacteria: e.g., genome surveillance, response to cell wall stresses, and peptidoglycan homeostasis

(ZEEVI *et al.*, 2013). Considering that bacteria use diverse cyclic nucleotide (or dinucleotide) messengers such as c-di-GMP (which in turn is related to mdrM and mdrT activity) to regulate basic processes like virulence, DNA damage, cell wall stress, adhesion and biofilm formation (ZEEVI *et al.*, 2013), it is to be expected the activity of MDR genes in *L. monocytogenes* biofilm formation tests in the presence (or not) of curing salts and quaternary ammonium compounds as observed in this study (Table 3).

Drug efflux is a crucial determinant contributing to both the intrinsic and/or acquired form of antimicrobial resistance and may involve flexible and overlapping drug specificities (AZIZOGLU *et al.*, 2015). Among transporters harbored by *L. monocytogenes* strains, energy-dependent major facilitator super family (MFS) efflux system transporter proteins such as Lde have been implicated in disinfectant resistance (RAKIC-MARTINEZ *et al.*, 2011).

Strains of *L. monocytogenes* with genotypic resistance to QACs may have mutations that lead to a reduction in cell permeability. For instance, resistant strains may have modifications in membrane fatty acids and phospholipids, which can lead to amore anionic and hydrophobic cell surface. This makes it difficult the QAC´s passage through the membrane and entry in the cell. In other cases, resistance to QAC's in *L. monocytogenes* may be due to the acquisition of QAC-specific efflux pumps through recombinant elements and mobile genetic elements (RAKIC-MARTINEZ *et al.*, 2011). Resistance also may be due to the over expression of endogenous efflux pumps due to mutations in regulatory elements. This can occur due to exposure to QACs or the stress induced by these compounds. These pumps are usually chromosomally encoded and affect a broad spectrum of antimicrobial compounds. In certain strains of *L. monocytogenes* that are resistant to sanitizers, QAC resistance has been associated with the over expression of efflux pumps in the MFS (major facilitator superfamily) group, such as *Lde* (*Listeria* drug efflux) (RAKIC-MARTINEZ *et al.*, 2011). The stress survival islet (SS-1) represented in this study by the genes *Lmo0444*, *Lmo0445* and *Lmo0446*, has been considered to influence biofilm formation and is also implicated in the survival of *L. monocytogenes* under stressful conditions (adaptation to low pH and high salt concentration) in food processing environments (Keene et al., 2018). The ability of *L. monocytogenes* to adpate and to survive under stressful conditions such as extreme temperatures, alkaline pH and presence of ethanol was associated with the ability to form biofilms, although the SS-1 has a clear role in survival under stressful conditions, its performance in the ability of adhesion and subsequent *L. monocytogenes* biofilm formation is little known (KEENEY et al., 2018).

Keeney *et al.*, (2018) analyzed biofilm data from 166 environmental and food related *L. monocytogenes* isolates by WGS and revealed that serotypic and genetic factors that strongly correlate with adherence and biofilm formation, such as lineage, plasmid harbouring and the presence of the stress survival islet 1 (SSI-1). The results also showed that strains from serotype 1/2b, the majority of which contained SSI-1, formed the strongest biofilms, while serotype 4b strains, the majority of which did not contain SSI-1, formed the weakest biofilms. When serotype 1/2a was separated by its SSI-1 genotype, SSI-1-positive 1/2a strains demonstrated significantly higher capacity for biofilm formation after 72 hours of growth at 30 °C. Together, these findings indicate that SSI-1 may contribute to serotype-associated differences in the biofilm-forming capacity in *L. monocytogenes*.

4 CONCLUSION

Collected data allowed to identify the ability of *L. monocytogenes* isolates from the meat processing environment to form biofilm in stainless steel. Despite of the stressing conditions applied (7.5% cure salt and quaternary ammonium (1: 1,024) in the biofilm formation at (37 °C / 72 h) being determinant for biofilm reduction, isolates previously characterized as possessing strong adhesion potential (lineage I) and persistent (lineage II) were able to form biofilm in the presence of these stressful compounds. These findings supporting the ability of these strains to remain in the meat processing environment by forming biofilms and to overpass stressful conditions.

The qPCR analysis allowed to predict the expression of genes previously related to initial attachment to abiotic surfaces (*flaA*), action in the early stages of biofilm formation (*agrB* and *agrC*) and resistance to salts (*agrC*), multi-drug transporter genes (*mdrT* and *mdrM*), resistance to quaternary ammonium by efflux pumps (*ldE*), adaptation to low pH and high salt concentration (*Lmo0444*, *Lmo0445*, *Lmo0446*), highlighting this genetic machinery in biofilm formation in stainless steel surfaces under the presence of curing salts 7.5%, quaternary ammonium (1: 1024) and even in the control situation BHI broth (37 °C / 72 h).

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Bibliography

AZIZOGLU, R. O. *et al.* Resistance of *Listeria monocytogenes* biofilms to sanitizing agents. Biofilms in the Food Environment, p. 51, 2015.

BUCHANAN, R. L.; PHILLIPS, J. G. Response surface model for predicting the effects of temperature pH, sodium chloride content, sodium nitrite concentration and atmosphere on the growth of *Listeria monocytogenes*. Journal of Food Protection, v. 53, n. 5, p. 370–376, 1990.

CABEÇA, T. K. Suscetibilidade de microorganismos relacionados com a contaminação de alimentos em biofilme artificial e em suspensão frente a desinfetantes. 2006.

CABEÇA, T. K.; PIZZOLITTO, A. C.; PIZZOLITTO, E. L. Activity of disinfectants against foodborne pathogens in suspension and adhered to stainless steel surfaces. Brazilian Journal of Microbiology, v. 43, n. 3, p. 1112–1119, 2012.

CARANDINA, D. C. F. Avaliação de biofilmes formados por isolados de *Listeria monocytogenes* provenientes de laticínios e perfil de resistência a agentes sanitizantes. . [S.l.]: Universidade de São Paulo. , 2013

CHMIELEWSKI, R. A. N.; FRANK, J. F. Biofilm formation and control in food processing facilities. Comprehensive Reviews in Food Science and Food Safety, v. 2, n. 1, p. 22–32, 2003.

FARBER, J. M.; PETERKIN, P. I. *Listeria monocytogenes*, a food-borne pathogen. Microbiological Reviews, v. 55, n. 3, p. 476–511, 1991.

GANDRA, T. K. Valente *et al.* Expression levels of the agr locus and prfA gene during biofilm formation by *Listeria monocytogenes* on stainless steel and polystyrene during 8 to 48 h of incubation 10 to 37° C. International Journal of Food Microbiology, v. 300, p. 1–7, 2019.

GUESCINI, M. *et al.* A new real-time PCR method to overcome significant quantitative inaccuracy due to slight amplification inhibition. BMC Bioinformatics, v. 9, n. 1, p. 326, 2008.

HERIGSTAD, B.; HAMILTON, M.; HEERSINK, J. How to optimize the drop plate method for enumerating bacteria. Journal of Microbiological Methods, v. 44, n. 2, p. 121–129, 2001.

KEENEY, K *et al.* Stress survival islet 1 contributes to serotype-specific differences in biofilm formation in Listeria monocytogenes. Letters in Applied Microbiology, v. 67, n. 6, p. 530–536, 2018.

LEMON, K. P.; HIGGINS, D. E.; KOLTER, R. Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. Journal of Bacteriology, v. 189, n. 12, p. 4418–4424, 2007.

LERICHE, V.; CARPENTIER, B. Viable but nonculturable *Salmonella typhimurium* in single-and binary-species biofilms in response to chlorine treatment. Journal of Food Protection, v. 58, n. 11, p. 1186–1191, 1995.

LIU, D. Preparation of *Listeria monocytogenes* specimens for molecular detection and identification. International Journal of Food Microbiology, v. 122, n. 3, p. 229–242, 2008.

MACKEY, B. M. *et al.* Heat resistance of *Listeria*: strain differences and effects of meat type and curing salts. Letters in Applied Microbiology, v. 10, n. 6, p. 251–255, 1990.

MAFRA, I. *et al.* Comparative study of DNA extraction methods for soybean derived food products. Food Control, v. 19, n. 12, p. 1183–1190, 2008.

MINEI, C. C. *et al.* Influence of peroxyacetic acid and nisin and coculture with Enterococcus faecium on *Listeria monocytogenes* biofilm formation. Journal of Food Protection, v. 71, n. 3, p. 634–638, 2008.

NORWOOD, D. E.; GILMOUR, A. The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. Journal of Applied Microbiology, v. 88, n. 3, p. 512–520, 2000.

OUYANG, Y. *et al.* Genome-wide screening of genes required for *Listeria monocytogenes* biofilm formation. Journal of Biotech Research, v. 4, p. 13, 2012.

PAN, Y.; BREIDT, F.; KATHARIOU, S. Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. Applied and Environmental Microbiologia, v. 72, n. 12, p. 7711–7717, 2006.

PEANO, C. *et al.* Qualitative and quantitative evaluation of the genomic DNA extracted from GMO and non-GMO foodstuffs with four different extraction methods. Journal of Agricultural and Food Chemistry, v. 52, n. 23, p. 6962–6968, 2004.

PIETA, L. *et al.* Transcriptional analysis of genes related to biofilm formation, stress-response, and virulence in Listeria monocytogenes strains grown at different temperatures. Annals of Microbiology, v. 64, n. 4, p. 1707–1714, 2014.

POIMENIDOU, S. V. *et al.* Variability of *Listeria monocytogenes* strains in biofilm formation on stainless steel and polystyrene materials and resistance to peracetic acid and quaternary ammonium compounds. International Journal of Food Microbiology, v. 237, p. 164–171, 2016.

PÖNTINEN, A. *et al.* Screening of the two-component-system histidine kinases of *Listeria monocytogenes* EGD-e. LiaS is needed for growth under heat, acid, alkali, osmotic, ethanol and oxidative stresses. Food Microbiology, v. 65, p. 36–43, 2017.

RAKIC-MARTINEZ, M. *et al. Listeria monocytogenes* strains selected on ciprofloxacin or the disinfectant benzalkonium chloride exhibit reduced susceptibility to ciprofloxacin, gentamicin, benzalkonium chloride, and other toxic compounds. Applied and Environmental Microbiology, v. 77, n. 24, p. 8714–8721, 2011.

STEINER, A. E.; MARAGOS, M. M.; BRADLEY JR, R. L. Cleanability of stainless steel surfaces with various finishes. Dairy, Food and Environmental Sanitation, v. 20, n. 4, p. 250–260, 2000.

THÉVENOT, D.; DERNBURG, A.; VERNOZY-ROZAND, C. An updated review of *Listeria monocytogenes* in the pork meat industry and its products. Journal of Applied Microbiology, v. 101, n. 1, p. 7–17, 2006.

VATANYOOPAISARN, S. *et al.* Effect of flagella on initial attachment of *Listeria monocytogenes* to stainless steel. Applied and Environmental Microbiology, v. 66, n. 2, p. 860–863, 2000.

WINKELSTRÖTER, L. K. *et al. Lactobacillus sakei* 1 and its bacteriocin influence adhesion of *Listeria monocytogenes* on stainless steel surface. Food Control, v. 22, n. 8, p. 1404–1407, 2011.

WINKELSTRÖTER, L. K.; DE MARTINIS, E. C. P. Different methods to quantify *Listeria monocytogenes* biofilms cells showed different profile in their viability. Brazilian Journal of Microbiology, v. 46, n. 1, p. 231–235, 2015.

YANG, H. *et al.* Efficacy of sanitizing agents against *Listeria monocytogenes* biofilms on high-density polyethylene cutting board surfaces. Journal of Food Protection, v. 72, n. 5, p. 990–998, 2009.

ZEEVI, M. K. *et al. Listeria monocytogenes* multidrug resistance transporters and cyclic di-AMP, which contribute to type I interferon induction, play a role in cell wall stress. Journal of Bacteriology, v. 195, n. 23, p. 5250–5261, 2013.

ZETZMANN, M. *et al.* Characterization of the biofilm phenotype of a *Listeria monocytogenes* mutant deficient in agr peptide sensing. Microbiology Open, p. e826, 2019.

Figure 1. Absorbance values recorded from *L. monocytogenes* isolates subjected to adhesion potential test in stainless steel microtitre plate, co-inoculated with disinfetant A (1:1024), NaCl and cure salts concentrations, submitted to incubation for 72 h at 37 °C.

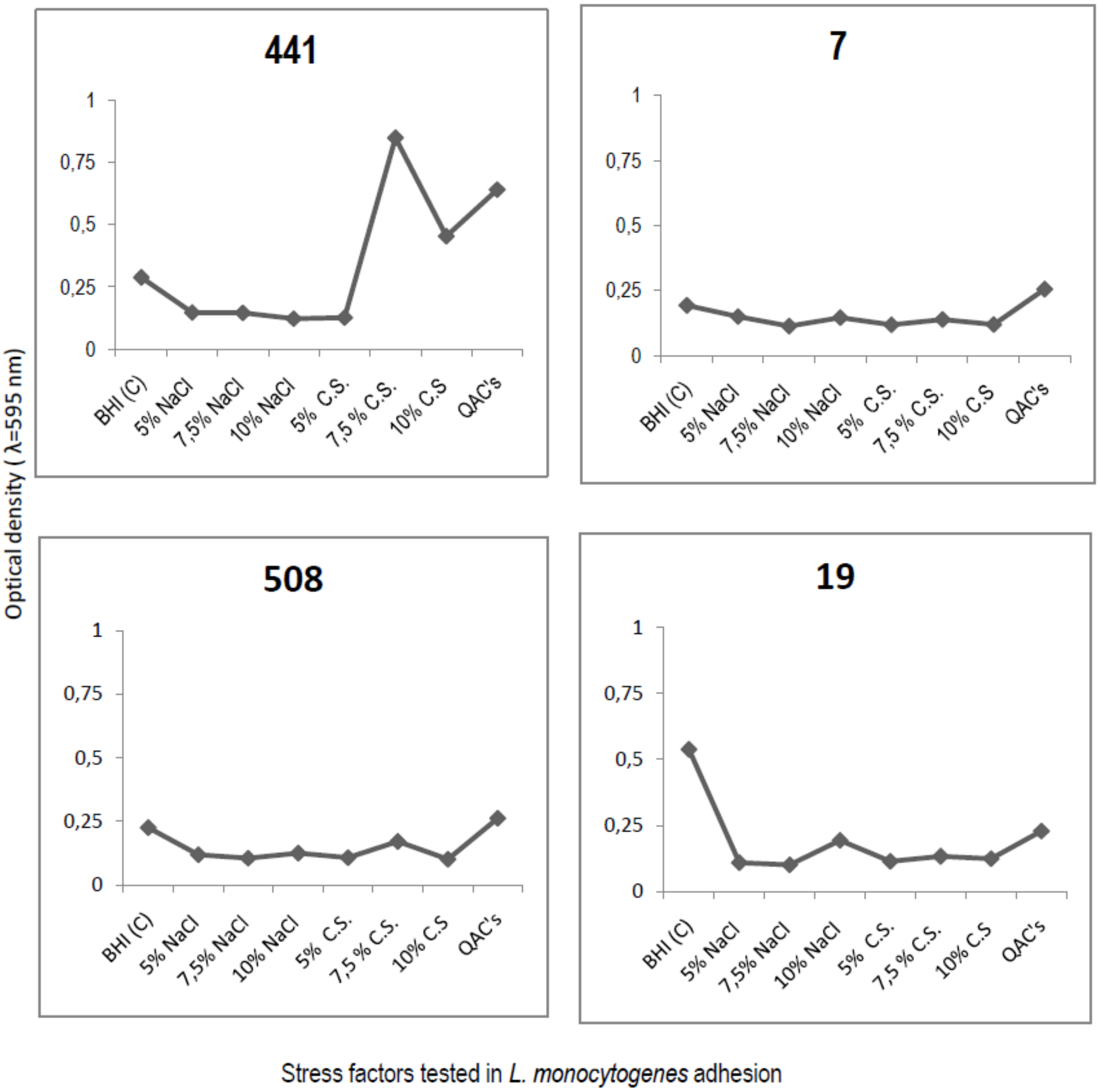

Table 1. Origin description and genotypes of isolates used in the present study, genotyped previously by Camargo *et al*., (2019).

Isolate	Year	State	Source type	Sample type		Serotype	PCR-serogroup[3]	Lineage	CC (MLST)[4]	ST (MLST)[4]	SL (cgMLST)[5]	CT (cgMLST)[5]
19	2009	MG[1]	PE[2]	Meat handlers	Before processing	1/2c	IIc	II	CC9	ST9	SL9	CT4420
508	2012	MG[1]	Food	Raw beef	Refrigerated	1/2c	IIc	II	CC9	ST9	SL9	CT4420
CLIST 441	2010	MT	Food	Raw beef	Refrigerated	1/2b	IIb	I	CC3	ST3	SL3	CT4447
7	2009	MG	Food	Raw beef	Refrigerated	1/2b	IIb	I	CC3	ST3	SL3	CT4448

MG, Minas Gerais; MT, Mato Grosso;

[1] Recovered from the same food processing plant.

[2] Production environment.

[3] According to Doumith *et al*., (2004) and Leclercq *et al*., (2011).

[4] Clonal complex (CC) and sequence type (ST) defined according to Ragon *et al*., (2008).

[5] Sublineage (SL) and cgMLST type (CT) defined according to Moura *et al*., (2016).

Table 2. Panel of selected genes related to the biofiilm formation and environmental stress resistance used in qPCR assays.

Gene	Reference	Sequence	Productlenght	Function
rplD1	Miranda *et al*., (2017)	F: 5-GTCCCTTGACGTAGGGATGC-3 R: 5-GGAACAAACGCTGGCGAAAT-3	113 bp	Normalizer
flaA	Pieta *et al*., (2014)	F: 5-GTAAGCATCCAAGCGTCTGA-3 R: 5-AAGAATCAGCATCAGCAACG-3	148 bp	Influences biofilm formation
agrB	Autret *et al*., (2003)	F: 5-AGGTACATTTGGATTTATACTGCTCAAC-3 R: 5-TCTTCACCGATTAAAGGCAAACT-3	81 bp	Adaptation to environmental conditions
agrC	Autret *et al*., (2003)	F: 5-ATTGACAAGATTTCGATGGATAGTATAGATT-3 R: 5-CACAAGTTAACGCCGCTTCA-3	88 bp	Adaptation to environmental conditions
mdrT	Kaplanzeevi *et al*., (2013)	F: 5-CCGTGCGGTTCTTCGGTAT-3 R: 5-TTTACTGCCGAACCGTGGTT-3	100 bp	Multidrug resistance
mdrM	Kaplanzeevi *et al*., (2013)	F: 5- CAGCAAGTACATCAGTGAAGCGTAA-3 R: 5- GGTAGCGCGACATTCATCAA-3	100 bp	Resistence to sanitezer quaternary ammonium
ldE	Tamburro *et al*., (2015)	F: 5- GGCACTATCAACGGCAGCGGT-3 R: 5- TGTGTCCGACAACGCTCCACC-3	70 bp	*Listeria* drug efflux
SSI-1 *lmo 0444*	Ryan *et al*., (2010)	F: 5-CATCTGCTCTTGTCGGTTCA-3 R: 5-CCGACACCATTCTCAAGGTT-3	85 bp	Adaptation to low pH and high salt concentration
SSI-1 *lmo 0445*	Ryan *et al*., (2010)	F: 5-TAGACGAGCTTTGGAACCTC-3 R: 5-GGTATCGGGGCCATTTCTTC-3	99 bp	Adaptation to low pH and high salt concentration
SSI-1 *lmo 0446* (*pva*)	Ryan *et al*., (2010)	F: 5-TTGCGCAACGATTAAAGATG-3 R: 5-TCACTACACAACGCCCACTC-3	131 bp	Adaptation to low pH and high salt concentration

Figure 2. Lineage I *L. monocytogenes* biofilm enumeration CFU/ cm² in two stainless steel systems (microplate and coupons).

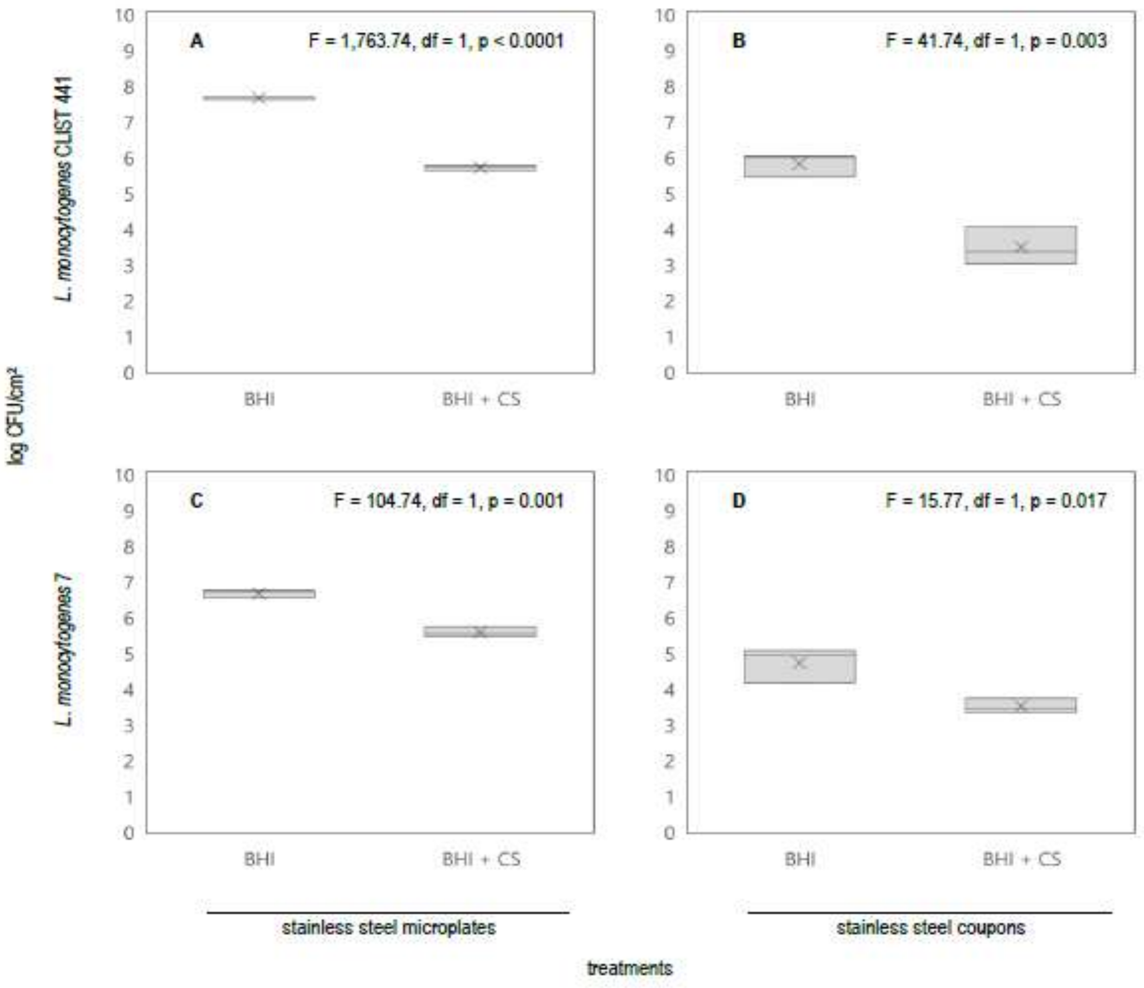

Figure 3. Lineage II *L. monocytogenes* biofilm enumeration CFU/ cm² in two stainless steel systems (microplate and coupons).

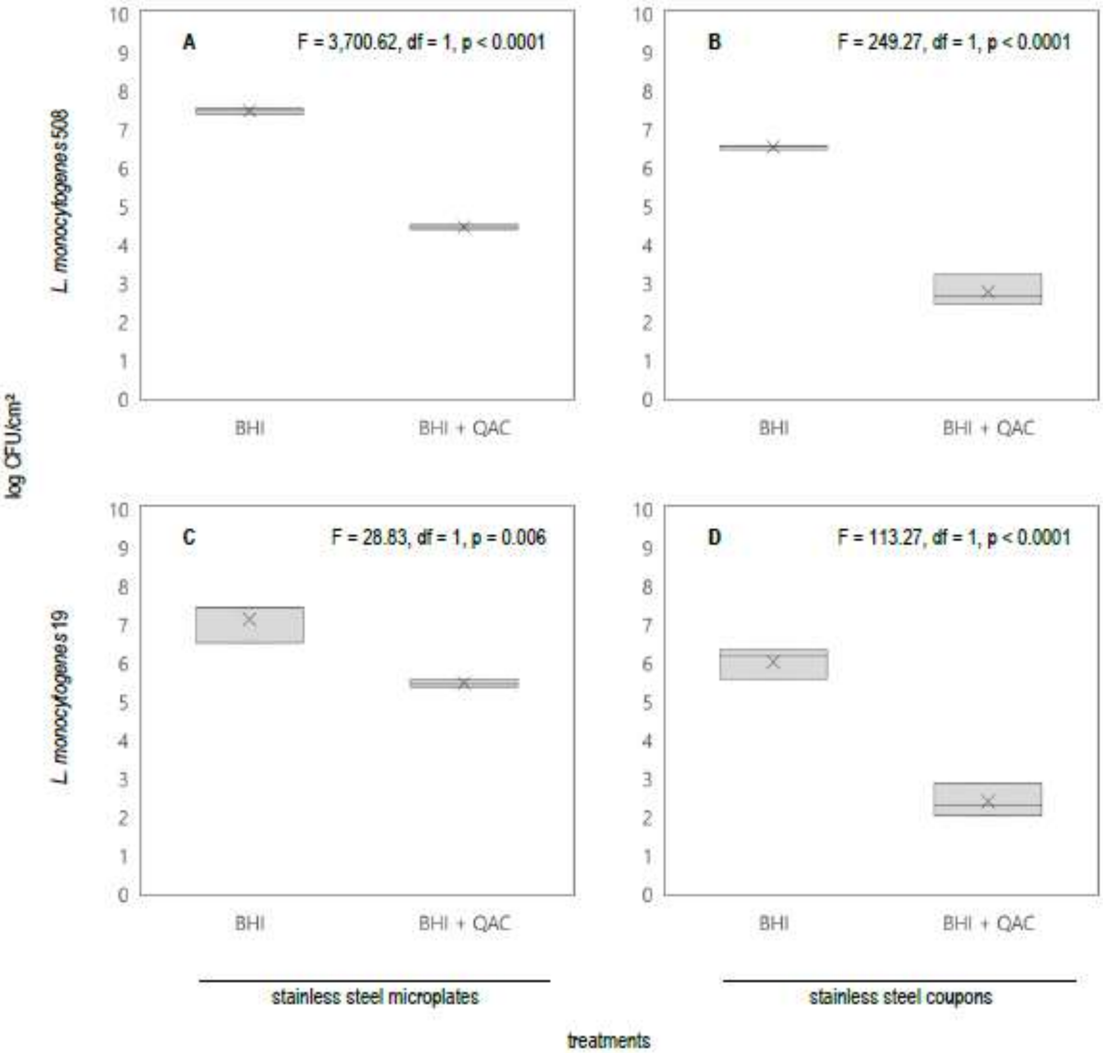

Table 3. Positivity panel according to eleven selected genes on gene expression prediction by qPCR.

Treatment	Isolate	UFC/cm²	*rlpD1*	*flaA*	*agrB*	*agrC*	*mdrT*	*mdrM*	*ldE*	*Lmo0444*	*lmo0445*	*lmo0446*
BHI	CLIST 441	2.6 10^5	🟦	🟥	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟥
BHI	CLIST 441	9.7 10^5	🟦	🟥	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟥
BHI + C.S	CLIS T441	2.2 10^3	🟦	🟥	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟥
BHI + C.S	CLIST 441	1.1 10^4	🟦	🟥	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟥
BHI	7	1.1 10^5	🟦	🟥	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟥
BHI	7	8.6 10^4	🟦	🟥	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟥
BHI + C.S	7	5.2 10^3	🟦	🟥	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟥
BHI + C.S	7	2.8 10^3	🟦	🟥	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟥
BHI	508	3.6 10^6	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
BHI	508	3.4 10^6	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
BHI+QAC	508	2.6 10^2	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
BHI+QAC	508	4.2 10^2	🟦	🟦	🟦	🟦	🟦	🟦	🟥	🟦	🟦	🟦
BHI	19	3.6 10^5	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
BHI	19	1.4 10^6	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
BHI+QAC	19	7.1 10^2	🟦	🟦	🟦	🟥	🟦	🟦	🟦	🟦	🟦	🟦
BHI+QAC	19	1 10^2	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦

🟦 Positive gene expression 🟥 Impossble to predict gene expression

Table 4. qPCR parametrers used to identify *L. monocytogenes* biofilm gene expression.

Primer	Isolate (Control DNA)	Amplicon size	Control Melt Temperature °C Values	Control CycleThereshold Values	*L. monocytogenes* biofilm (cDNA) Cycle Thereshold Values Variation
rlpD1	CLIST 441	113 bp	82	13.06	12.18 to 25.15
rlpD1	7	113 bp	82.2	22.03	
rlpD1	508	113 bp	82	13.06	
rlpD1	19	113 bp	82	15.06	
flaA	CLIST 441	148 bp	81.5	18.02	8.09 to 13.14
flaA	7	148 bp	81	15.98	
flaA	508	148 bp	81	8.05	
flaA	19	148 bp	81	11.01	
agrB	CLIST 441	81 bp	80.7	12.05	8.09 to 13.1
agrB	7	81 bp	80.2	15.98	
agrB	508	81 bp	80.5	12.62	
agrB	19	81 bp	80.3	13.06	
agrC	CLIST 441	88 bp	78.7	15.06	8.13 to 21.11
agrC	7	88 bp	78.5	13.98	
agrC	508	88 bp	78.5	8.05	
agrC	19	88 bp	78.7	12.05	
mdrT	CLIST 441	100 bp	83	25.15	9.13 to 14.9
mdrT	7	100 bp	82.2	30	
mdrT	508	100 bp	82.5	27.04	
mdrT	19	100 bp	83.2	30	
mdrM	CLIST 441	100 bp	80.7	12.01	7.05 to 18.74
mdrM	7	100 bp	80.5	10.05	
mdrM	508	100 bp	81	9.05	
mdrM	19	100 bp	80.8	13.02	
ldE	CLIST 441	70 bp	83.5	13.02	12.09 to 19.46
ldE	7	70 bp	83.7	12.01	
ldE	508	70 bp	84.5	15.06	
ldE	19	70 bp	84.5	16.14	
Lmo0444	CLIST 441	85 bp	78.5	12.18	7.17 to 16.22
Lmo0444	7	85 bp	79	12.05	
Lmo0444	508	85 bp	78.7	9.01	
Lmo0444	19	85 bp	78.7	12.01	
Lmo0445	CLIST 441	99 bp	80	9.97	10.01 to 13.34
Lmo0445	7	99 bp	79	9.05	
Lmo0445	508	99 bp	79.2	8.01	
Lmo0445	19	99 bp	79.3	10.05	
Lmo0446	CLIST 441	131 bp	82.5	8.99	3.04 to 16.06
Lmo0446	7	131 bp	81.8	10.01	
Lmo0446	508	131 bp	82	8.05	
Lmo0446	19	131bp	82	10.29	

SUPPLEMENTARY MATERIAL

Table S1. Presence or absence of genes related to the biofilm formation and environmental stress resistance analysis of the selected isolates.

Gene	Locus-tag	Function*	Strains			
			7	*CLIST 441*	*19*	*508*
comK	-	Biofilm formation and virulence	+	+	-	-
esaA	lmo0757	Enhance biofilm formation at 15 °C	+	+	+	+
-	lmo0453	Enhance biofilm formation at 15 °C	+	+	+	+
-	lmo0543	Enhance biofilm formation at 15 °C	+	+	+	+
uvrA	lmo2488	Enhance biofilm formation at 15 °C	+	+	+	+
-	lmo1224	Enhance biofilm formation at 15 °C	+	+	+	+
-	lmo2563	Enhance biofilm formation at 15 °C	+	+	+	+
-	lmo2572	Enhance biofilm formation at 15 °C	+	+	+	+
flaA	lmo0690	Influences biofilm formation	+	+	+	+
luxS	lmo1290	Influences biofilm formation	+	+	+	+
rmlB	lmo2229	Influence biofilm formation	+	+	+	+
uvrB	lmo2489	Influence biofilm formation	+	+	+	+
mgtB	lmo2689	Influence biofilm formation	+	+	+	+
murA	lmo2526	Alter biofilm architecture	+	+	+	+
degU	lmo2515	Regulation of motility, adherence to plastic surfaces and biofilm formation	+	+	+	+
-	*lmo0444*	Adaptation to low pH and high salt concentration	+	+	+	+
-	*lmo0445*	Adaptation to low pH and high salt concentration	+	+	+	+
pva	lmo0446	Adaptation to low pH and high salt concentration	+	+	+	+
gadD1	lmo0447	Adaptation to low pH	+	+	+	+
gadT1	lmo0448	Adaptation to low pH	+	+	+	+
-	lin0464	Adaptation to alkaline and oxidative stress	-	-	-	-
-	lin0465	Adaptation to alkaline and oxidative stress	-	-	-	-
cspD	lmo1879	Cold adaptation and oxidative stress tolerance	+	+	+	+
cspB	lmo2016	Cold adaptation and oxidative stress tolerance	+	+	+	+
lisK	lmo1378	Growth and adaptation at low temperature, stress tolerance and virulence	+	+	+	+
lisR	lmo1377	Growth and adaptation at low temperature, stress tolerance and virulence	+	+	+	+
oppA	lmo2196	Growth at low temperature and intracellular survival	+	+	+	+
agrB	lmo0048	Adaptation to environmentalconditions	+	+	+	+
agrD	lmo0049	Adaptation to environmental conditions	+	+	+	+
agrC	lmo0050	Adaptation to environmental conditions	+	Mutation	+	+
agrA	lmo0051	Adaptation to environmental conditions	+	+	+	+
rsbR	lmo0889	Stress adaptation	+	+	+	+
rsbS	lmo0890	Stress adaptation	+	+	+	+
rsbV	lmo0893	Stress adaptation	+	+	+	+
rsbX	lmo0896	Stress adaptation	+	+	+	+
perR	lmo1683	Stress-regulator	+	+	+	+
mdrM	Lmo1617	Resistence to sanitizer quaternary ammonium	+	+	+	+
mdrT	-	Efflux pump	+	+	+	+
mdrL	-	Multidrug resistant *Listeria*	+	+	+	+
Lde	Lmo2741	*Listeria* drug efflux	+	+	+	+
emrE	-	Resistence to quaternary ammonium sanitizers	-	-	-	-

*References: (RABINOVICH *et al.*, 2012; VERGHESE *et al.*, 2011); (PIERCEY; HINGSTON; HANSEN, 2016); (LEMON; HIGGINS; KOLTER, 2007); (TODHANAKASEM; YOUNG, 2008); (VATANYOOPAISARN *et al.*, 2000); (BELVAL *et al.*, 2006); (SELA *et al.*, 2006); (CHANG *et al.*, 2012; OUYANG *et al.*, 2012); (NOWAK *et al.*, 2017); (MACHATA *et al.*, 2005); (PIERCEY; HINGSTON; HANSEN, 2016); (GUERIRI *et al.*, 2008); (KNUDSEN; OLSEN; DONS, 2004); (COTTER *et al.*, 2005); (FEEHILY *et al.*, 2014); (RYAN *et al.*, 2010); (HARTER *et al.*, 2017); (LOEPFE *et al.*, 2010); (SCHMID *et al.*, 2009); (WEMEKAMP-KAMPHUIS *et al.*, 2002); (COTTER *et al.*, 1999; PÖNTINEN *et al.*, 2015); (AUTRET *et al.*, 2003); (GARMYN *et al.*, 2009); (VIVANT *et al.*, 2014); (GARMYN *et al.*, 2012); (RIEDEL *et al.*, 2009); (RIEU *et al.*, 2007); (VIVANT *et al.*, 2015); (MARTINEZ; REEVES; HALDENWANG, 2010); (WIEDMANN *et al.*, 1998); (CHATURONGAKUL; BOOR, 2004); (GIOTIS *et al.*, 2010); (KAZMIERCZAK *et al.*, 2003); (HUANG *et al.*, 2018; (RAKIC-MARTINEZ *et al.*, 2011; (QUILLIN; SCHWARTZ; LEBER, 2011); (KOVACEVIC *et al.*, 2016).