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ANIMAL SCIENCE

Detection of virulence genes and antimicrobial susceptibility profile of *Listeria monocytogenes* isolates recovered from artisanal cheese produced in the Southern region of Brazil

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Abstract: *Listeria monocytogenes* is an opportunistic pathogen that causes listeriosis, a foodborne disease with low incidence but with high mortality rate in humans. This microorganism has been recovered from several dairy products, especially those produced with raw milk. The objective of this work was to investigate the presence of virulence genes, and also to define the antimicrobial susceptibility profile of *L. monocytogenes* isolates recovered from serrano artisanal cheese produced in Southern region of Brazil. Nine strains of *L. monocytogenes* (serotypes 1/2b and 4b) were evaluated through PCR to detect the presence of the virulence genes *hly*, *inlA*, *inlC*, *inlJ*, *actA*, *plcB* and *iap*, while antimicrobial susceptibility profile was determined via disk diffusion method. All strains exhibited the presence of the genes *hly* and *plcB*, whereas the other genes (*iap*, *actA*, *inlA*, *inlC* and *inlJ*) were only detected in eight strains. We verified that all strains were resistant to at least one antimicrobial agent and three of them showed multidrug resistance. These findings demonstrated the serrano artisanal cheese offers risks to consumers' health and point to a need of adaptations and monitoring of manufacturing process of this food, in order to prevent the dissemination of *L. monocytogenes*.

Key words: dairy products, multidrug resistance, raw milk, virulence genes.

INTRODUCTION

Listeria monocytogenes is a pathogen that causes listeriosis, a serious foodborne disease that can be fatal. The overall mortality rate is estimated in 30%, especially in those with underlying diseases and/or immunosuppressed status, elderly and fetus or neonate (Drevets & Bronze 2008, Lecuit 2007). *Listeria monocytogenes* is a concern in the food industry, since it is found in various environments, and it grows under different conditions, such as low temperatures, high salt concentrations and wide pH range (from 4.5 to 9.0) (Guenther et al. 2009, Walker et al. 1990).

This microorganism has been recovered from several types of food, among which serrano artisanal cheese (Melo et al. 2013, Pontarolo et al. 2017), a product typically manufactured in the highland fields the South of Brazil with raw milk obtained from dairy cattle subjected to natural grazing-pasture management (Pereira et al. 2014). The raw milk contaminated by *L. monocytogenes* introduced into the food processing environment and used to manufacture unpasteurized cheese is a serious threat to human health, due to

the specific abilities of this pathogen to resist numerous stresses (Kousta et al. 2010). For that reason, its control continues to be a challenge for food processing (Melo et al. 2015).

There are thirteen recognized serotypes of *L. monocytogenes*, which are distributed into four genetic lineages (Orsi et al. 2011), being more than 95% of human listeriosis commonly associated with isolates that belong to serotypes 4b, 1/2a and 1/2b (Doumith et al. 2004, Kasper et al. 2009). The pathogenicity of *L. monocytogenes* is determined by several virulence factors, such as: listeriolysin O (LLO), internalins, phospholipases, actin assembly-inducing protein (ActA), invasion-associated protein (p60) and regulatory system for gene expression of virulence (PrfA) (Liu 2006).

Listeria monocytogenes is usually susceptible to several antimicrobial agents. However, recent studies have shown an emergence of isolates recovered from food samples, which are resistant to antibiotics commonly used for human listeriosis therapy (Fallah et al. 2013, Kevenk & Gulel 2016, Noll et al. 2018, Tahoun et al. 2017). This fact is of great concern, since *L. monocytogenes* is able to develop resistance mechanisms or to acquire resistance through transmission of genetic material of other bacterial species at the food-processing environment (Allen et al. 2016, Bertsch et al. 2013, Toomey et al. 2009).

Therefore, the objective of the present study was to investigate the presence of virulence genes and to define the antimicrobial susceptibility profile of *L. monocytogenes* isolates obtained from serrano artisanal cheese samples manufactured in the South region of Brazil, Santa Catarina State.

MATERIALS AND METHODS

Bacterial isolates

A total of nine strains of *Listeria monocytogenes*, serotypes 1/2b (n=2) and 4b (n=7), recovered from serrano artisanal cheese were used in this study. Strains were isolated in previous investigations performed by Melo et al. (2013) and Pontarolo et al. (2017) at Centro de Diagnóstico Microbiológico Animal (CEDIMA) of Universidade do Estado de Santa Catarina (UDESC), in partnership with serrano artisanal cheese producers from Santa Catarina, southern region of Brazil. In these studies, a total of 170 cheeses were analyzed and *L. monocytogenes* was isolated in nine cheeses (5.29%).

Bacterial DNA extraction

Isolation of genomic DNA from recovered and quality control strains (*L. moncytogenes* ATCC *EGD-e* and *E. coli* ATCC 25922) was performed from the protocol described by Doyle & Doyle (1987) with some modifications. Bacterial strains were cultivated in Brain Heart Infusion (BHI) broth for 24 h at 37ºC. A total of 200µL of each inoculum was transferred to a sterile microtube. 500µL of chloroform: isoamyl alcohol (24:1) were added and this mixture was further incubated in a water-bath for 30 min at 56ºC. After that, microtubes were centrifuged for 10 min at 12,000 rpm. The retrieved supernatant was transferred to another sterile microtube and 600µL of 70% alcohol were added. This mixture was centrifuged for 20 min at 13,500 rpm. The supernatant was discarded by inversion, and pellet was allowed to air-dry. The dried pellet was resuspended with 200µL of sterile Milli-Q water.

Virulence genes detection

The presence of seven genes encoding virulence factors of *L. monocytogenes* were investigated in this study: internalin A (*inlA*), internalin C

(*inlC*), internalin J (*inlJ*), actin assembly-inducing protein (*actA*), phospholipase C - PC-PLC (*plcB*), listeriolysin O, invasion-associated protein - p60 (*iap*). Multiplex PCR described by Liu et al. (2007) was used to investigate the presence of virulence genes *inlA*, *inlC* and *inlJ*, whereas an adaptation of Cao et al. (2018)'s protocol was carried out for the detection of the other genes (*actA, plcB, hly* and *iap*).

PCR reaction was carried out in 25 µL final volume of reaction mixture that contained PCR buffer (Tris-HCl - 20mM, KCl - 50mM), $MgCl_2$ (2mM), dNTP (200mM of each), *Taq* DNA polimerase (0.5U), primers (4 pmol of each) and bacterial DNA (2µl). Amplification was performed using a denaturation step of 94°C for 4 min, followed by 32 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 60 s. A final extension step at 72°C for 10 min was applied. Amplicons were electrophoresed (100V, 300mA) on 2% agarose gel for 1h. After that, we stained the agarose gel with GelRed™ and amplified fragments were visualized on transilluminator (Kasvi, model K33-312, Brazil). The used primers are listed on Table I. All reagents were purchased from Invitrogen® (Carlsbad, USA) and reactions were carried out in Thermal Cycler Applied Biosystem (model MJ96, Thermo Fisher, USA). Quality control strains *Listeria monocytogenes EGD-e* and *Escherichia coli* ATCC 25922 were used as positive and negative controls, respectively.

Determination of antimicrobial susceptibility profile

The antimicrobial susceptibility test of the strains was carried out according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) using disk diffusion method in Mueller-Hinton agar supplemented with 5% defibrinated horse blood and 20 mg/L β-NAD (EUCAST 2018). The strains were tested for their susceptibility to the following antimicrobial

Table I. List of primers used to detect virulence genes in *Listeria monocytogenes* isolates.

Gene	Oligonucleotide sequence (5'-3')	Size of amplicon (bp)	Virulence factor	Described by:
inlA	F: ACGAGTAACGGGACAAATGC R: CCCGACAGTGGTGCTAGATT	800	Internalin A	Liu et al. (2007)
inlC	F: AATTCCCACAGGACACAACC R: CGGGAATGCAATTTTTCACTA	517	Internalin C	
inlJ	F: TGTAACCCCGCTTACACAGTT R: AGCGGCTTGGCAGTCTAATA	238	Internalin J	
actA	F: TGCATTACGATTAACCCCGACA R: AGGCTTTCAAGCTCACTATCCG	431	Actin assembly-inducing protein	Cao et al. (2018)
plcB	F: AGTGTTCTAGTCTTTCCGG R: ACCTGCCAAAGTTTGCTGT	792	Phospholipase C (PC-PLC)	
hly	F: ACGCAGTAAATACATTAGTG R: AATAAACTTGACGGCCATAC	372	Listeriolysin O	
iap	F: TTTGCTAAAGCGGGTATCTC R: AGCCGTGGATGTTATCGTAT	205	Invasion-associated protein (p60)	

agents: benzylpenicillin (PEN, 1unit), ampicillin (AMP, 2µg), meropenem (MER, 10µg), erythromycin (ERI, 15µg) and trimethoprim/sulfamethoxazole (TMP/SMX 1.25 / 23.75 µg). Results of growth inhibition zone were interpreted according to current EUCAST (2018) guidelines established for *L. monocytogenes*. Besides that, ciprofloxacin (CIP, 5µg), levofloxacin (LVX, 5µg), gentamicin (GEN, 10µg), clindamycin (CLI, 2µg), tetracycline (TET, 30µg), chloramphenicol (CLO, 30µg) and rifampicin (RIF, 5µg) were also tested. However, interpretation of these results were performed based on the critical points recommended by EUCAST (2018) for *Staphylococcus* spp., since there are not established breakpoints of these antibiotics for *L. monocytogenes*. We used *Streptococcus pneumoniae* ATCC 49619 and *Staphylococcus aureus* ATCC 29213 as quality control strains to define antimicrobial susceptibility profile.

RESULTS AND DISCUSSION

A total of nine strains of *L. monocytogenes* recovered from artisanal cheese, classified as 1/2b (n=2) and 4b (n=7) serotypes, were analyzed for the presence of seven virulence genes. The low number of strains analyzed is a limitation of this study, since its occurrence in foods is rare. The genes *hly* and *plcB* were detected in all strains, whereas the others (*iap*, *actA*, *inlA*, *inlC* e *inlJ*) were found in eight strains (Table II). Coroneo et al. (2016) reported similar results in *L. monocytogenes* isolates from cheese in Italy, with variable rates of virulence gene detection. In addition, other studies also demonstrated similar finding in samples obtained from different types of food, raw milk, milking machine, worker's hands and clinical specimens (Du et al. 2017, Su et al. 2016, Tahoun et al. 2017). However, studies based on several types of food have documented the presence of virulence genes in all examined *L. monocytogenes* isolates (Almeida et al. 2017, Iglesias et al. 2017, Jamali et al. 2013, Mammina et al. 2009, Oliveira et al. 2018).

It is known that some polymorphisms and punctual mutations, present in certain virulence genes, could contribute for attenuated-virulence from *L. monocytogenes* strains (Orsi et al. 2011, Van Stelten et al. 2010). Based on that, absence or presence of virulence factors could be a tool to assess not only the risks related with food product consumption, but also those associated with strain-specific virulence parameters of *L. monocytogenes* (Jacquet et al. 2004).

We findings that eight strains were positive for all virulence factors investigated (Table II). The pathogenicity of *L. monocytogenes* is defined by several virulence factors, in particular the family of internalins, bacterial surface proteins responsible for internalization (entry) of *L. monocytogenes* into cells (InlA, InlB) (Bonazzi et al. 2009, Hamon et al. 2006); and dissemination between cells (InlC) (Rajabian et al. 2009). Another crucial role is played by listeriolysin O (LLO), a protein responsible for survival and intracellular multiplication of this pathogen, since it allows bacterial phagosomal escape to the cytoplasm of infected host cells (Hamon et al. 2012, Ruan et al. 2016). Based on that, the *L. monocytogenes* replicates, but intracellular movement and cell-to-cell dissemination will only occur with help of ActA protein, which will induce polymerization of actin and, consequently, actin-filament formation inside of the host cells (Cameron et al. 1999, Kocks et al. 1995). In addition to these virulence factors, we should also mention the phospholipases C, such as PC-PLC (PlcB), that acts in synergy with LLO to amplify phagocytic vacuole's lysis (Camilli et al. 1993, Gedde et al. 2000); and the protein (p60)

Table II. Profile of virulence and antimicrobial susceptibility of *Listeria monocytogenes* isolates recovered from serrano artisanal cheese.

Isolates	*Serotype*	*Virulence genes* *							*Antimicrobial resistance* **
		hly	*inlA*	*inlC*	*inlJ*	*actA*	*plcB*	*iap*	
Lm1	1/2b	+	-	-	-	-	+	-	TMP/SMX, CLI, RIF
Lm2	4b	+	+	+	+	+	+	+	TMP/SMX, CIP, CLI, RIF
Lm3	4b	+	+	+	+	+	+	+	TMP/SMX, CLI
Lm4	1/2b	+	+	+	+	+	+	+	CLI, LVX
Lm5	4b	+	+	+	+	+	+	+	TMP/SMX, RIF
Lm6	4b	+	+	+	+	+	+	+	TMP/SMX, CLI
Lm7	4b	+	+	+	+	+	+	+	CLI
Lm8	4b	+	+	+	+	+	+	+	CIP, CLI
Lm9	4b	+	+	+	+	+	+	+	ERI, TMP/SMX, CIP, CLI, LVX, TET, RIF

*** (+) = presence; (-) = absence. ** CIP – ciprofloxacin; CLI – clindamycin; ERI – erythromycin; LVX – levofloxacin; RIF – rifampicin; TET – tetracycline; TMP/SMX – trimethoprim/sulfamethoxazole.**

encoded by the gene *iap*, which is necessary for a successful invasion (Camejo et al. 2011).

The presence of the virulence genes demonstrate the potential pathogenicity of these strains. This finding provides evidence of a serious health public issue, since these strains represent a potential threat (Listeriosis) to serrano artisanal cheese consumers, especially those from the risk groups (pregnant women, elderly and immunocompromised individuals).

Regarding antimicrobial susceptibility, *L. monocytogenes* isolates had their sensitivity to a panel of 12 antibiotics investigated. All strains were resistant to at least one antimicrobial agent, while three of them exhibited a multidrug resistance profile (resistance to three or more antimicrobial agent classes) (Table II). Our results are in line with previous studies that demonstrated isolation rates ranging from 21% to 88% of multidrug resistant *L. monocytogenes* recovered from different types of food, among them raw milk and raw dairy products (Fallah et al. 2013, Harakeh et al. 2009, Kevenk & Gulel 2016, Noll et al. 2018, Tahoun et al. 2017). *L. monocyotogenes* strains were more frequently resistant to clindamycin, trimethoprim/sulfamethoxazole, rifampicin and ciprofloxacin. On the other hand, all strains were susceptible to ampicillin, benzylpenicillin, meropenem, gentamicin and chloramphenicol (Table III). Similarly, Tahoun et al. (2017) evaluated patterns of antimicrobial resistance of *L. monocytogenes* isolates, recovered from raw milk, milking equipment, and hand swabs collected in Egyptian dairy farms. The authors observed a high resistance rate of these isolates to clindamycin and rifampicin. Furthermore, recent studies carried out worldwide (Brazil, China and Spain) detected high prevalence (ranging from 35 to 81%) of resistant *L. monocytogenes* isolates to clindamycin, which were recovered from different types of food (Du et al. 2017, Gómez et al. 2014, Oliveira et al. 2018). In relation to other agents, there are reports about high prevalence of *L. monocytogenes* isolates, recovered from different types of food, to sulfonamide in Brazil

Table III. Antimicrobial susceptibility profile of *L. monocytogenes* isolates from serrano artisanal cheese.

Antimicrobial agents	*No. of isolates (%)*	
	Resistant (R)	*Susceptible (S)*
Ampicillin (2µg)	-	9 (100)
Benzylpenicillin (1unit)	-	9 (100)
Meropenem (10µg)	-	9 (100)
Erythromycin (15µg)	1 (11.1)	8 (88.9)
Trimethoprim/sulfamethoxazole (1.25/23.75µg)	6 (66.7)	3 (33.3)
Ciprofloxacin (5µg)	3 (33.3)	6 (66.7)
Levofloxacin (5µg)	1 (11.1)	8 (88.9)
Gentamicin (10µg)	-	9 (100)
Clindamycin (2µg)	8 (88.9)	1 (11.1)
Tetracycline (30µg)	1 (11.1)	8 (88.9)
Chloramphenicol (30µg)	-	9 (100)
Rifampicin (5µg)	4 (44.5)	5 (55.5)

(Iglesias et al., 2017) and to ciprofloxacin in Egypt (El Banna et al. 2016) and China (Wang et al. 2013).

However, most of the resistance detected in the *L. monocytogenes* strains was against antimicrobials without breakpoints for this microorganism and the interpretation parameters used were the breakpoints for *Staphylococcus* spp.

Studies reported the ability to transfer antimicrobial resistance genes between *L. monocytogenes* and other microorganisms in food matrices such as milk and cheese (Bertsch at al. 2013, Toomey et al. 2009). This fact emphasizes a critical problem in the food industry, since *L. monocytogenes* strains endures in food processing plants persistently for years or even decades (Ferreira et al. 2014). Survival or growth of this microorganism on surfaces of difficult hygienization in the food processing environment could be related to this endurance, or also the repeatedly reintroduction of this pathogen from external to processing environment through raw material, equipment or production workers (Buchanan et al. 2017). Therefore, serrano artisanal cheese-processing plants require especial attention, since this cheese is a raw milk product and its production and storage (depends on maturation period) are commonly precarious, which means that do not follow the established hygienic-sanitary guidelines.

Contaminated environments can create optimal conditions for different types of bacteria to promote genetic material exchange, particularly for those products that require extended period of preparation and storage. Although resistance mechanisms for *L. moncytogenes* are still not well established, it is plausible that this microorganism acquires and disseminates mobile genetic elements that codify antimicrobial resistance genes derived from other pathogens in the food processing environment (Allen et al. 2016, Bertsch et al. 2013, Toomey et al. 2009).

CONCLUSIONS

In conclusion, our study clearly showed that *L. monocytogenes* isolates from serrano artisanal cheese revealed the presence of the major virulence factors involved in its pathogenicity. Besides that, antimicrobial susceptibility investigation exhibited a high index of resistance to multiple agents used for human listeriosis treatment. Consequently, it is important to develop strategies to face efficiently microbial safety challenges and to monitor strictly the raw milk cheese processing. By that, we will not only prevent dissemination of this microorganism, but we will also reassure that this product does not offer risks to consumers' health.

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Leandro Parussolo conceived the study, designed the experiments, coordinated the investigation, analyzed the data and wrote the paper. Ricardo Antônio Pilegi Sfaciotte, Karine Andrezza Dalmina and Fernanda Danielle Melo performed laboratory analysis and drafted the experimental section. Ubirajara Maciel da Costa and Sandra Maria Ferraz critically reviewed the manuscript. All the authors have read and approved the final manuscript.