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Review scientific paper

ECOLOGY OF LISTERIA MONOCYTOGENES

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Summary

Food safety criteria for *Listeria monocytogenes* in the ready-to-eat food category have been applied since 2006. In the territory of the European Union in 2019, there were 2 621 confirmed cases of invasive listeriosis in humans, with a high mortality rate (17.6%).
A broader understanding of the ecology of *Listeria monocytogenes* is crucial for the successful control of this pathogen in the food chain continuum.
The paper presents new knowledge on reservoirs/sources of contamination, distribution, dynamics and transmission routes, survival and persistence of *Listeria monocytogenes*, both in natural habitat and in farm or processing environment.
Keywords: *Listeria monocytogenes*, ecology, zoonoses

INTRODUCTION

Listeria monocytogenes survives in various habitats (natural - soil, surface water, decaying vegetation; farm environment and processing environment), but is also recognized as a cause of human and animal infections. According to the latest Report on Zoonoses (EFSA, 2021), there was 2 621 confirmed cases of invasive listeriosis in humans in the European Union in 2019, with a high mortality rate (17.6%). Of particular importance is the fact that hypervirulent strains of *Listeria monocytogenes* are isolated from contaminated food.
The ubiquitous nature of this opportunistic pathogen as well as the presence of numerous growth niches in the production environment can establish persistent, so-called. "In-house" strains *of Listeria monocytogenes*, when food contamination occurs during and after production. As *Listeria monocytogenes* is inactivated by heat treatment regimes applicable in the production of ready-to-eat food, the subsequent, so-called postprocessing contamination is most important. In the control of this type of contamination, different approaches are applied: strict and consistent application of measures of good hygienic and good manufacturing practice, standard sanitation work procedures, aseptic packaging technology, so-called "post-lethal" treatments during the packaging phase or reformulation of the composition of the product in such a way that the food matrix does not support the growth of *Listeria monocytogenes*, and/or the addition of an antimicrobial agent. However, for successful control of this pathogen in the food chain, a more complete understanding of the ecology of *Listeria monocytogenes* is crucial. The application of newer molecular methods of subtyping, which are based on

genome sequencing, enables the assembly of the whole, providing objective data on the distribution of *Listeria spp*. as well as the survival and persistence of *Listeria monocytogenes* in different ecosystems, sources of contamination, dynamics and transmission pathways.

Dr Jekyll and Mr Hyde „personality" of *Listeria monocytogenes*

Gray et al. (2006) have described the double life of *Listeria monocytogenes*as Dr. Jekyll and Mr Hyde's personality. *Listeria monocytogenes* saprophytes in soil and decaying vegetation (*Dr Jekyll*), while in its second life, as an intracellular pathogenic bacterium it can cause serious infections in humans and many species of animals (*Mr Hyde*). Transformation from one phase to another is mediated by complex regulatory pathways, which modulate the expression of virulence factors in response to changes in the immediate environment of *Listeria monocytogenes*. Little is known about the non-harmful existence of *Listeria monocytogenes*. Genome sequencing indicates many gene products that allow the utilization of various carbon sources, including plant sugars. Access to nutrient sources is facilitated by flagella expression as well as mobility at temperatures below 30°C (repression of this activity occurs at 37°C). The robust nature of *Listeria monocytogenes* is also reflected in its ability to survive in unfavorable environmental conditions (low temperature, high osmolarity, increased NaCl concentration).

It is possible that the existence *of Listeria monocytogenes* outside mammalian host cells does not mean exclusively and completely peaceful "rural" life, but a constant territorial battle with other unicellular and multicellular organisms, which "lurk" in the environment. Although *Listeria monocytogenes* is commonly isolated from environmental sources, the bacterium maintains a large arsenal of genes whose products allow bacteria to survive within the mammalian host cell. The maintenance of these genes in ubiquitous organisms suggests that the role of these genes is not exclusively invasion and survival within mammalian cells, but also possible interaction with other eukaryotic organisms in the environment, which could serve as the primary reservoir of *Listeria monocytogenes*. So far, such a reservoir has not been identified, although it is thought that protozoa (*Acanthamoeba spp*.) may be a link between the ecology and pathology of *Listeria monocytogenes* (Schuppler, 2014). *Acanthamoeba spp*. have been studied for many years as a model of a eukaryotic cell. They feed on smaller organic particles, bacteria, fungi, algae, and even other protozoa. They are considered the main consumers of bacteria, responsible for the reduction (up to 60%) of the total bacterial population in soil and other natural ecosystems. Due to their similarity to macrophages, free-living amoebae are considered a potential reservoir of *Listeria monocytogenes*, where, figuratively speaking, listeria would serve as a training ground for development of pathogenesis (intracellular survival and proliferation). However, the results of many studies show that *Listeria monocytogenes* does not survive within the phagosome of *Acanthamoeba spp*. and thus these protozoa cannot play the role of reservoirs of *Listeria monocytogenes*. The paradox is that although Listeria monocytogenes does not survive digestion by *Acanthamoeba spp*., growth in the co-culture of these two entities promotes

the growth of listeria. It is hypothesized that *Listeria monocytogenes* has the ability to utilize metabolites secreted by *Acanthamoeba spp*. Bearing in mind the possible synergistic effect, with the fact that amoebae are isolated from cucumbers, cabbage, lettuce, celery, carrots, radishes, mushrooms, cauliflower and spinach, and that a rich community of protozoa (amoebae, ciliates and flagellates) is determined in the processing environment (meat processing plants) (Vaerewijck et al., 2008), but also in households (refrigerators) (Vaerewijck et al., 2010), it is clear that this type of interaction can have significant implications for food safety and human health.

The expression of those *Listeria monocytogenes* genes responsible for the mechanism of pathogenesis (invasion, survival, and replication within the host cell, including macrophages and nonprofessional phagocytes) is regulated by a transcriptional activator known as positive regulatory factor A (PrfA) (Milohanic et al., 2003). *Listeria monocytogenes* strains, which lack functional PrfA, show impaired virulence (attenuation) in animal models. It is known that PrfA exists in high and low activated states, and the transition from one state to another, which corresponds to the activation or repression of transcription, is conditioned by signals from the environment. Growth in a nutrient-rich medium, or in a medium enriched with readily available carbohydrates (glucose, fructose, cellobiose - a common carbohydrate of plants), inhibits the transcription of PrfA - dependent virulence genes. In contrast, phosphorylated sugars, present in the cytosol of mammalian cells, as well as low iron content, support the growth of *Listeria monocytogenes*, without inhibiting the expression of virulence factors.

Outside the host organism of mammals (eukaryotic cells), *Listeria monocytogenes* maintains the personality of *Dr. Jekyll* by repressing PrfA production and activity through appropriate transcriptional, post-transcriptional, and post-translational mechanisms. At the moment when the bacterium is ingested by the mammalian host, elevated temperature and exposure to low pH values (gastric juice), stimulates increased production of stress response proteins, internalin and PrfA, which causes the transition to *Mr Hyde* (virulent stage). In the gut, internalin A mediates epithelial cell adhesion and invasion. The low concentration of iron and carbohydrates in the phagosome vacuole causes repression of internalin production, but promotes PrfA-dependent activation of promoters responsible for the production of cytolysin listeriolysin, which leads to lysis of the phagocytic vacuole and transition of listeria to cytosol cells. The cytosol completes the complete transformation of *Dr Jekyll* into *Mr Hyde* personality, where a high level of active PrfA protein allows transcription of appropriate promoters, resulting in the production of ActA protein and phospholipase, or intracellular motility and spread of listeria to surrounding cells.

Genetic population structure of *Listeria monocytogenes*

Listeria monocytogenes inhabits various ecological niches (natural habitat, farm environment, animals, food, people), and survives over a long period of time in unfavorable environmental conditions. For these reasons, it is very difficult to identify sources of contamination and monitor transmission routes, and the epidemiology of many cases of human listeriosis remains unclear. Fortunately, the development and application

of molecular methods of subtyping allows the generation of large amounts of data, whose analysis and proper interpretation provide a full picture of the ecology of *Listeria monocytogenes*. Methods commonly used in the typing of *Listeria monocytogenes* isolates include ribotyping and Pulsed Field Gel Electrophoresis. In these methods, restriction endonucleases are used to digest bacterial DNA and generate fragments of appropriate size (*DNA fragment-based subtyping methods*). Among other things, despite the existence of data analysis software, these methods are very difficult to standardize. In addition, although used for cluster analysis, they generally do not provide information on the primary genetic characteristics (nucleotide sequence) of isolates, and the application of these methods is not justified for the phylogenetic analyzes. In DNA sequencing-based methods, the full or partial nucleotide sequence is determined for one (*single locus approach*) or more bacterial genes or chromosomal regions. By applying these methods, objective data are obtained that can be used to determine evolutionary relatedness (phylogeny). Of these methods, the most commonly used is the *Multi Locus Sequence Typing* (MLST) method, as well as the *Multilocus Enzyme Electrophoresis* (MLEE). More recently, *Whole Genome Sequencing* (WGS), in particular the sequencing of multiple loci within the genome that is shared by all strains of one species and is not susceptible to the mutations (core genome MLST- cgMLST), is used in the epidemiological typing of *Listeria monocytogenes*.

Molecular analysis of a large number of isolates from different sources and geographical areas over the last 90 years confirms the genetic heterogeneity of *Listeria monocytogenes* and emphasizes the clonal structure of the population. The current classification includes four major phylogenetic lines (I-IV) (lineages), more than 14 serotypes (Rodriguez et al., 2021) and four PCR serogroups (multiplex polymerase chain reaction for amplification of 4 specific marker genes; correlation with *Listeria monocytogenes* serotypes: 1/2a-3a; 1/2b-3b-7; 1/2c-3c and 4b-3b-7). Each of the lines implies specific serotypes. Line I contains serotypes 1/2b, 3b, 4b, 4d, 4e and 7, line II serotypes 1/2a, 1/2c, 3a and 3c, line III serotypes 4b, 1/2a, 4a and 4c and line IV serotypes 4a and 4c. Serotypes of line III and IV are rarely isolated and are mainly detected in ruminants. Most isolates detected in human listeriosis form a cluster within line I (serotypes 1/2b, 3b, 4b) and line II (serotypes 1/2a, 1/2c, 3a), with a clear dominance of serotypes 4b (most clinical isolates), 1/2a (largest number of isolates originating from the processing environment) and 1/2b. These are genetically related strains, ie specific clonal groups, globally distributed, which, according to one system of nomenclature, are presented as epidemic clones. Epidemic Clones (ECs) are defined as a group of genetically related isolates implied in cases of either a single epidemic or geographically and temporally unrelated cases of listeriosis epidemics, and originated from a common ancestor (Cantinelli et al., 2013). So far, 11 epidemic clones (ECI - ECXI) have been identified (Filipello et al., 2017). The second system of nomenclature represents clonal complexes (CC), based on the application of MLST typing. This method of genotyping is based on differences in the nucleotide sequences of seven constitutively expressed genes (*housekeeping genes*), which encode enzymes responsible for different types of intermediate metabolism of bacteria. *"Housekeeping"* genes are slowly changing and the results obtained are more important in

population genetics than in epidemiology. In the case when non-housekeeping genes are selected for sequencing, MLST is also used in epidemiological typing. With this typing approach, each recognized allele in the pubMLST database is assigned an allelic number, and based on a combination of seven allelic numbers, the database recognizes one type of nucleotide sequence, which is referred to as the *Sequence Type* (ST). This methodology classifies strains into sequential types (unique association of alleles from seven housekeeping genes), ie clonal complexes, which include a group of two or more independent isolates that divide the same alleles into five or more gene loci (clusters of sequential types), and originate from the same ancestor. Sequence types (ST) are placed in the appropriate clonal complex by the BURST (Based Upon Related Sequence Types) analytical program. BURST analysis first determines the groups of related genotypes in the population, presented in the pubMLST database and tries to identify the founding genotype, ie. ST of each group. The algorithm then predicts the descendants of the founding genotype, displaying the result in the form of a radial diagram with the predicted founder in the center of the circle. The definition of a group starts from the assumption that each ST coincides with another ST in the group in relation to five or more loci.

The results of the studies show that clinical isolates and isolates of *Listeria monocytogenes* originating from food represent separate, although often overlapping populations (Gray et al., 2004). The differences between these two populations are primarily manifested in the potential for virulence. The internalin A protein, encoded by the inlA gene, is a significant virulence factor for *Listeria monocytogenes*, responsible for intestinal epithelial cell invasion. An interesting observation is the fact that mutations are found in *Listeria monocytogenes* isolates, which result in the synthesis of a shortened form of this protein (premature termination of amino acid chain elongation due to premature insertion of a termination stop codon into the nucleotide sequence of a structural gene) ie *truncated protein*. Isolates of *Listeria monocytogenes*, which carry these mutations, are characterized by impaired virulence and are commonly detected in serotypes of line II, 1/2a, 1/2c, which is a possible explanation why the same serotypes are far less isolated in clinical cases of human listeriosis. Although serotypes 1/2a and 1/2c are more often isolated from food, it would be wrong to conclude that, by consuming contaminated food, people are exclusively exposed to a subpopulation of attenuated listeria. Namely, virulent strains, including epidemic, hypervirulent clones, are also isolated from the food matrix.

The use of molecular subtyping methods identifies hypervirulent MLST clones of *Listeria monocytogenes* with a high clinical frequency (CC1, CC2, CC4 and CC6) (Maury et al., 2016). Isolates of *Listeria monocytogenes* belonging to CC1 complex in high prevalence are determined in cases of rhomboencephalitis in ruminants (Dreyer et al., 2016), while analysis of feces of cattle and sheep shows prevalence of 4b serotype (CC1 complex) (Esteban et al., 2009). The presented results show not only the high virulence of this clone in relation to cattle, but well argue the fact that cattle can serve as a significant reservoir of hypervirulent clones of *Listeria monocytogenes* and disperse them through feces in the farm environment. In contrast, CC9 and CC121 originate from food, are

hypovirulent, and cause infections mainly in highly immunocompromised human subpopulations, which is at least partially explained by mutations that ultimately result in the synthesis of the non-functional protein internalin A. In addition, CC121 has been found to persist in food production facilities (Ortiz et al., 2016).

A very interesting study was conducted by Maury et al. (2019). The authors tried to find a link between individual clones (CC) of *Listeria monocytogenes* and certain food categories. The results of this study showed that hypervirulent clones (CC1, CC4 and CC6) are associated with dairy products, while hypovirulent clones (CC9 and CC121) are isolated from meat products and show a higher prevalence of stress resistance and tolerance to benzalkonium chloride. The observed difference in the distribution of clones in relation to the food category indicates differences in the modality of contamination (milk vs meat), as well as the diversity of the niche (adaptation of clones to different environmental conditions). The meat is initially physiologically sterile, and contamination of meat products with *Listeria monocytogenes* occurs during processing and storage. Milk contamination occurs before (primary contamination) and/or during milking (secondary contamination). This hypothesis is supported by the observation that clones CC9 and CC121 represent the least represented clones in raw milk products, while in pasteurized milk they represent the second (CC9) and seventh (CC121) most common clones. Hypervirulent clones better colonize the intestinal lumen and invade host tissues, indicating that the same clones are adapted to the host organism. In contrast, hypovirulent clones are adapted to a processing environment where they can persist for a long time based on greater resistance to stress and tolerance to benzalkonium chloride, but also the ability to form a biofilm at sublethal concentrations of benzalkonium chloride. In addition to hyper and hypovirulent clones, intermediate clones (CC2 and CC6) are isolated from the food matrix, which are in transition from the stage of adaptation to the host to the saprophytic life form, loss of virulence factors, and the adoption of genes responsible for tolerance to disinfectants. In addition, in hypovirulent clones, a higher prevalence of genes involved in the processes of replication, recombination and repair of genetic damage is determined, which indicates a possible greater exposure of these clones to genotoxic conditions. On the other hand, the higher prevalence of genes involved in the processes of cell wall and membrane biogenesis in hypervirulent clones of *Listeria monocytogenes* indicates the possible selection of those genes that are responsible for the interaction with the host.

Listeria monocytogenes in natural habitats

Ever since the pioneering work of Welshimer (1960), there has been a consensus of the scientific community that land represents a niche of strong importance in the transmission of *Listeria monocytogenes* to plants and animals. Soil is composed of organic matter, minerals, plant roots and complex biota, including microorganisms, viruses, mesofauna and macrofauna. All these factors are interconnected and in constant interaction, and the land represents a complex environment in dynamic equilibrium. For this reason, identification of those factors that determine the prevalence of *Listeria monocytogenes* in soil is very difficult. Soil composition, microbial communities and macrofauna, free

water availability, and, according to recent research (Locatelli et al., 2013; McLaughlin et al., 2011) especially soil pH, are intrinsic factors determining the fate of *Listeria monocytogenes* in soil. Agricultural practices (ensiling of contaminated crops, recycling of organic fertilizers without previously applied sanitation procedures), meteorological conditions, proximity to surface waters, dairy farms, roads and urban areas are extrinsic factors. The plasticity of the genome of *Listeria monocytogenes*, with a wide repertoire of genes encoding transport and regulatory proteins, indicating a huge ability to adapt and persist this pathogen, is an additional factor responsible for the habitat of *Listeria monocytogenes* in soil. A large part of the genes (26%), which encode transport proteins, are directed to the transport of carbohydrates via phosphoenolpyruvate-dependent phosphotransferase systems. This enables the use of different carbon sources, which is an advantage and greater competitiveness in the conditions of selective pressure that is inevitably established in such densely populated ecosystems. Wild animals, including mammals and birds, can be considered a potential zoonotic reservoir of *Listeria monocytogenes* and play a role in the transfer of this microorganism to the soil. The land of cultivated fields and pastures can serve as a vector of *Listeria monocytogenes* on cultivated crops and farm animals, especially cattle and small ruminants, which then serve as a significant reservoir of listeria.

Although the results of the conducted studies indicate a general presence of *Listeria monocytogenes* in soil samples, the level of contamination is low (Locatelli et al., 2013; Dowe et al., 1997).

The ubiquitous nature of *Listeria monocytogenes* and *Listeria spp*., as well as the fact that surface watercourses are used to discharge wastewater from sewage, have resulted in the presence of these microorganisms in many surface waters, including lakes, rivers and streams. To date, epidemiological data do not confirm direct human infection through contaminated water, due to low levels of contamination, although contaminated surface water has a role in dispersing listeria to wider geographical areas.

The natural environment can serve as a source, although not necessarily as a reservoir of *Listeria monocytogenes* strains. Haydon et al. (2002) define the reservoir of infection as one or more epidemiologically related populations or environments in which the pathogenic microorganism is permanently maintained and from where the infection "spreads" to the target population. Although it seems possible that the natural environment, primarily land, is a reservoir for certain subtypes of *Listeria monocytogenes* that cause animal infections, it remains unclear whether ruminants are a true "target" population or listeria can be maintained in the ecosystem without causing animal infections. Namely, according to one hypothesis, *Listeria monocytogenes* can be a "random" pathogen of humans and farm animals (so-called "*dead-end*" hosts), which, in this case, is not particularly important for the survival of this species of microorganisms. According to the same scenario, the survival of *Listeria monocytogenes* in natural environments would include possible, until now unidentified hosts (protozoa, lower vertebrates), and existence in these hosts would be critical to the evolutionary success of this bacterial species.

Listeria monocytogenes in silage

The results of numerous studies confirm the connection between silage diet and cases of listeriosis in cattle and sheep. Good quality silage, prepared from grass, corn, whole grains and legumes, establishes anaerobic conditions that promote the growth and reproduction of indigenously present or inoculated lactic acid bacteria (LAB). Optimal growth conditions enable a LAB number of 109 CFU g to be achieved within 48 hours. The metabolic activity of LAB converts plant sugars into lactic acid, which results in a decrease in pH (pH value of well-preserved silage is <4.5). Acidic environmental conditions inhibit the growth of spoilage microorganisms and *Listeria spp*. The optimal dry matter content in the ensiling mass is above 40% for legumes, about 30% for ensiling in the silo and about 40% in the bales. Silage with a high dry matter content has a higher pH value, but a lower content of free water in such silage inhibits the growth of listeria. Grass silage in colder, humid climates has a lower sugar content and higher water content, which can slow down the fermentation process, and such silage is more susceptible to growing listeria. The results of Dijkstra's (1971) study show that *Listeria monocytogenes* can survive four to six years in naturally contaminated silage.

Listeria monocytogenes is commonly found in baled silage due to high pH values and aerobic pockets, resulting from damage to the plastic sheath or insufficient number of sheath layers (Nucera et al., 2016). In order to prevent the risk of this type, it is necessary to use high-quality polyethylene stretch films, increase the number of layers of the coating, but also check the pH value before use for the purpose of contamination control (≤4-4.5). In a study by Pauly and Tham (2003), *Listeria monocytogenes* was not detected in untreated silage samples after 90 days of storage, even at pH 4.9 and above. The results of this study indicate that storage time may be one of the important factors for reducing the number of *Listeria monocytogenes* and in combination with optimal fermentation represents an effective measure in controlling the growth of *Listeria monocytogenes*. In the control of the growth of *Listeria monocytogenes* in silage, the addition of formic acid, lactic acid bacteria and bacteriocins, produced by *Streptococcus bovis* HC5 and *Pediococcus acidilactis* (Mantovani and Russell, 2003), has been successfully used.

Listeria monocytogenes on farms and in the farm environment

Excessive numbers of animals during the winter, when they stay in closed facilities, promote the spread of *Listeria monocytogenes* among animals, but also contamination of areas, including feeders, water troughs and agricultural land, where manure is used as organic fertilizer. The establishment of protection zones along surface watercourses in the immediate vicinity of the farm, within the farm itself, as well as buffer zones around the farm, has proven to be an effective measure in reducing contamination.

Water has been identified as a significant source of *Listeria monocytogenes* contamination on dairy farms. *Listeria monocytogenes* is found in pipes and water troughs (barns), drinking troughs, but also irrigation canals. By feeding on contaminated water, cows become listeria dispersal vectors on the farm and in the immediate vicinity of the farm, thus continuing the cycle of persistent bacterial infections.

As the soil is contaminated with *Listeria monocytogenes*, it is not surprising that the work boots of a farmer, veterinarian or farm visitor may play a role in dispersing bacteria (Schoder et al., 2013).

Hay, sawdust, fresh and recycled sand, calcium carbonate, and more recently recycled manure are used as animal bedding materials. Given the excretion of *Listeria monocytogenes* via faeces, in conditions of irregular fertilization, a high prevalence of *Listeria monocytogenes* in bedding material samples is expected (Bradley et al., 2018), although this contamination does not necessarily refer to the finding of *Listeria monocytogenes* in milk.

The existence of persistent niches of *Listeria monocytogenes* on the surfaces inside the farm (floors, feeding surfaces, water tank surfaces) has been described, which further increases the oral exposure of animals to this bacterium (Castro et al., 2018). Insufficient lighting of milking parlors, which prevents proper cleaning and disinfection, the ability of *Listeria monocytogenes* to create a biofilm on various surfaces, including plastic, rubber and stainless steel surfaces, especially on feeders, which are otherwise prone to damaging, but also on milking equipment further complicates the situation on the farm, and provides the possibility of establishing multiple sources of contamination.

In an extensive study by Latorre et al. (2009), strains of *Listeria monocytogenes* isolated from whole milk, milk system filters, milking equipment, cow feces, but also from mat samples, standing water, birds, bird droppings, wild animal droppings, insects, were subjected to molecular subtyping in order to identification of potential sources of contamination of whole milk. The test results indicate that the presence of *Listeria monocytogenes* in milk is initially caused by fecal or udder contamination, but then certain specific strains are isolated and permanently established in appropriate niches of the milking system in the form of biofilm, when continuous and repeated contamination of whole milk is possible.

Nightingale et al. (2004) hypothesize the following possible scenario for the transmission of *Listeria monocytogenes* on a dairy farm: initial contamination of crops and land through watercourses, wildlife, birds, and the use of manure to fertilize cultivated areas. Although farm animals can be directly exposed to *Listeria monocytogenes* from soil and crops during grazing, in this case, it is a low level of contamination, insufficient to cause infection. On the other hand, poor quality silage, inadequately preserved (pH> 5.05.5), and contaminated at the same time, allows *Listeria monocytogenes* to multiply to a high number and is most likely a common route of infection for farm animals. In the same study, a significantly higher prevalence of *Listeria monocytogenes* was found in fecal samples from dairy cows compared to samples from soil, water (farm environment) and animal feed. The authors, according to the obtained results, conclude that cattle, as hosts, amplify *Listeria monocytogenes* ingested through contaminated silage, and thus represent a critical factor in maintaining high levels of contamination on the farm. Scattering of *Listeria monocytogenes* through the feces of clinically infected animals and/or asymptomatic carriers, contamination of the immediate environment of the animals and the fecal-oral transmission cycle continues.

Milking and raw milk contamination

Primary milk contamination involves subclinical mastitis, where *Listeria monocytogenes* is identified as the causative agent. Although the prevalence of listerioid mastitis is low, and thus this time milk contamination is of less importance, Papić et al. (2019) shows the opposite. The results of the study indicate that subclinical mastitis cannot be neglected as a source of contamination of raw milk with *Listeria monocytogenes*, especially since molecular subtyping methods have determined that these isolates belong to hypervirulent clones CC2 and CC4. With the exception of this study, most authors agree that milk contamination with *Listeria monocytogenes* is mainly the result of secondary contamination (contamination of the udder surface with fecal material or from the environment), ie the result of biofilm formation in the milking system.

Listeria monocytogenes was detected in whole milk tanks and milking machines with a prevalence of 0.5% and at a contamination level <10 CFU/ml (Dalzini et al., 2016). Although the prevalence and concentration of *Listeria monocytogenes* is low, the presence of this bacterium in raw milk is still considered a risk to human health, especially if raw milk is intended for processing into products without heat treatment. Contamination of milk in the collection milk tank serves as a source of contamination for the rest of the milk that reaches the tank during milking.

Filters placed in the milking line are an important indicator of milk contamination with *Listeria monocytogenes*. Poor milking hygiene increases the amount of impurities on the filters, which favors the retention of *Listeria monocytogenes* in the impurity layer, and filters are considered far more sensitive indicators of the presence of bacteria in the milking system than whole milk samples (Giacometti et al., 2012). "Screening" of filters is a good method for identifying problems in hygiene and sanitation, not only at the level of milking but also in the immediate environment of animals. In a study obtained by Castro et al. (2018) *Listeria monocytogenes* was found in higher prevalence in filter samples than in whole milk samples, which indicates that low levels of contamination in whole milk samples go unnoticed, while the listeria is concentrated and detected at the filter level. Although filter screening increases the likelihood of detecting *Listeria monocytogenes* at the farm level, it is not a good indicator of the presence *of Listeria monocytogenes* in whole milk.

Listeria monocytogenes in processing environment

It is considered that the presence of *Listeria monocytogenes* in the process environment is the primary factor responsible for the subsequent, so-called postprocessing contamination during food production. *Listeria monocytogenes* has been found to persist for years or even decades in production facilities (Ferreira et al., 2014). In a broader sense, persistence is defined as the survival (usually without growth) of pathogenic microorganisms over a long period of time either in the food matrix or in a simple defined matrix (soil, water, stainless steel surfaces), but also in complex natural environments or environments generated by human activity (production plants). In practical conditions, persistence represents repeated isolation over a long period of time, and at different time intervals, of certain strains of *Listeria monocytogenes*, which are determined to belong to identical

subtypes using phenotypic and genetic methods. What is not clearly defined is the required frequency of repeated isolations, in order to differentiate the true persistence from the accidentally expected isolation. In addition, the method of subtyping, with limited power of discrimination, may not prove effective in determining the persistence of the same subtype through repeated sampling. Also, a major challenge is to distinguish true persistence from repeated reintroduction of certain *Listeria monocytogenes* subtypes into the production environment, which is not unexpected due to multiple sources and routes of contamination. Isolation of the same subtype through repeated sampling in the production plant, which uses controlled and microbiologically correct raw materials, applies the principles of hygienic design and good manufacturing practices, establishes rigorous cleaning and disinfection measures, very likely indicates true persistence. In contrast, in plants where the above measures are not observed, repeated isolation of the same subtype, especially in situations where limited methods of discriminatory power are used for subtyping, may indicate persistence, but also re-introduction of *Listeria monocytogenes* from the immediate production line environment. The basis of the persistence of certain subtypes of *Listeria monocytogenes* is the ability to create a biofilm, physiological tolerance to sanitation or barriers that are established during the process of food production and processing, or the appearance of a special subpopulation of the so-called persistent cells. Biofilm consists of cells and extracellular polymeric material, which protects cells from stress and promotes interactions between cells in relation to nutrients, toxic metabolites and genetic material, which ultimately results in improved survival and growth of bacterial cells. Although the results of many studies indicate that isolates of *Listeria monocytogenes*, depending on the strain, type of surface and temperature, show the ability to create a biofilm in the production plant, Ferreira et al. (2014) conclude that clear evidence of biofilm creation is lacking. It is possible that *Listeria monocytogenes* survives as part of a biofilm formed by several species of bacteria. One of the possible mechanisms of persistence of *Listeria monocytogenes* is tolerance to plant disinfectants. However, while some studies confirm that persistent strains are less sensitive to disinfectants, other studies do not find this type of correlation.

The dormant nature of persistent cells improves the ability of *Listeria monocytogenes* to survive under stress. Establishing a persistent subpopulation of *Listeria monocytogenes* is a strategy for long-term survival of listeria in unfavorable environmental conditions, and the transition to this condition is accompanied by a change in cell morphology - the transition from bacilli to cocci. Knudsen et al. (2013) report that a large number of strains of *Listeria monocytogenes*, isolated from clinical cases and epidemics of listeriosis, in response to antibiotics administered, show a biphasic population, characteristic of populations with persistent subpopulation. The same authors point out the possibility that the subpopulation of persistent cells enables the protection of *Listeria monocytogenes* during cleaning and disinfection in production facilities.

Study results of Magalhaes et al. (2016) showed that *Listeria monocytogenes* strains, frequently isolated during the four-year period from the cheese production plant, in conditions of high NaCl concentration and moderate acidity, are characterized by a shorter lag phase and faster growth rate, compared to sporadically isolated strains. The

difference in the sensitivity of these two groups of isolates of *Listeria monocytogenes*, in relation to sanitizers, was not observed.

CONCLUSION

Listeria monocytogenes is a ubiquitous bacterium with the ability to colonize many niches, but also a causative agent of serious infections in humans and animals. Dairy cow farms are recognized as a significant reservoir of *Listeria monocytogenes*, even those genotypes that are recognized as a causative agent in human listeriosis epidemics. The ecology of *Listeria monocytogenes* in a farm environment is very complex and, despite the efforts of the scientific community, insufficiently elucidated. The application of new methods of molecular subtyping (sequential methods) provides the possibility of determining the source of contamination and monitoring the transmission pathways of *Listeria monocytogenes*, in order to reduce the so-called contamination pressure at the farm level and established effective control measures

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