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CHARACTERIZATION OF MONOSPECIES BIOFILM CYCLE LIFE BY *LISTERIA INNOCUA*

Tesis en torno a una hipótesis o problema de investigación y su contrastación

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CHARACTERIZATION OF MONOSPECIES BIOFILM CYCLE LIFE BY *LISTERIA INNOCUA*

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DEDICATORIA

Quiero dedicar esta tesis primero a Dios por haberme permitido llegar hasta aquí y por darme salud y fortaleza para llevar a cabo mis metas y objetivos. A mi esposo por ser mi apoyo incondicional y mi compañero de vida. A mis padres por ser mi pilar en cada paso, por sus consejos, amor y apoyo.

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RESUMEN

Listeria innocua ha sido considerada a lo largo del tiempo una especie comensal y no patógena del género *Listeria,* aun así, existen reportes de infecciones causadas por *L. innocua* tanto en humanos como en animales. Se han reportado algunas especies atípicas con características hemolíticas, pero su potencial patógeno y su capacidad para persistir en el tiempo en forma de biofilms no se han caracterizado en detalle. En la revisión bibliográfica, reportamos las características genómicas y funcionales de las cepas hemolíticas de *L. innocua*, además de visibilizar los reportes del genoma completo donde se encuentran presentes varios factores de virulencia e islas de patogenicidad funcional en *L. innocua* iguales a las reportadas en *L. monocytogenes*.

Por otro lado, *Listeria innocua* ha sido detectada en plantas procesadoras de alimentos y se considera un problema de contaminación alimentaria. Nuestro objetivo fue cuantificar la formación de biofilms de *L. innocu*a monoespecies a 3 temperaturas diferentes y caracterizar su ciclo de vida en biofilms. Se evaluó la formación biofilms sobre vidrio en condiciones estáticas a 37, 22 y 4 ° C. Adicionalmente, se analizaron los biofilms a 37 ° C durante 12, 24, 48, 72, 80 y 96 horas mediante medición de violeta cristal, recuento de unidades formadoras de colonias (UFC) y microscopía fluorescente para el análisis de la viabilidad celular.

L. innocua demostró ser capaz de formar biofilms a las 3 temperaturas probadas, siendo 37 °C la temperatura optima. La producción de biofilms aumentó a las 72 horas hasta alcanzar un biofilm maduro y posteriormente disminuyo el recuento de UFC a las 96 horas. El análisis microscópico y la medición del cristal violeta mostraron un incremento de biomasa hasta las 96 horas. Nuestros resultados demostraron una gran adaptación de los biofilms de *L. innocua* a las condiciones térmicas y mostraron un ciclo de vida completo con 96 horas de crecimiento. Hasta donde sabemos, este es el primer estudio en Ecuador para evaluar la formación de biofilms de *L. innocua* por aislamientos de quesos frescos.

Palabras clave: *Listeria innocua*; genes de virulencia; formación de biopelículas; reportes del caso; esterilización en superficies**.**

ABSTRACT

Listeria innocua has been considered over time a non-pathogenic species, even so, there are reports of infections caused by *L. innocua* in both humans and animals. Some atypical species with hemolytic characteristics have been reported, but their pathogenic potential and their ability to persist over time in biofilms have not been characterized in detail. In the following bibliographic review, we report the genomic and functional characteristics of the hemolytic strains of *L. innocua*, in addition to making visible the reports of the complete genome where several virulence factors and islands of functional pathogenicity are present in *L. innocua* same as those reported. in *L. monocytogenes*.

On the other hand, *Listeria innocua* has been detected in food processing plants and is considered a food contamination problem. Our objective was to quantify the biofilm formation of *L. innocua* monospecies isolates from fresh cheeses at 3 different temperatures and to characterize their life cycle in biofilm. The ability of *L. innocua* to form biofilms on a glass surface was evaluated under static conditions at 37, 22 and 4°C. In addition, the biofilms were analyzed at 37°C for 12, 24, 48, 72, 80 and 96 hours by crystal violet measurement, counting of colony forming units (CFU) and fluorescent microscopy for analysis of cell viability.

L. innocua was able to form biofilms at 3 temperatures tested, but 37°C was the optimum temperature. Biofilm production increased at 72 hours until reaching a mature biofilm and subsequently decreased the CFU count at 96 hours. Microscopic analysis and measurement of crystal violet shows an increase in biomass up to 96 hours. Our results demonstrated a great adaptation of *L. innocua* biofilms to thermal conditions and showed a complete life cycle with 96 hours of growth. To our knowledge, this is the first study in Ecuador to evaluate the biofilm formation of *L. innocua* by isolates from fresh cheeses.

Key words: *Listeria innocua*; virulence genes; biofilm formation; case reports; sterilization on surfaces.

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PART I – STATE OF THE ART

LISTERIA INNOCUA NOT SO INNOCUA – A MINIREVIEW

Listeria innocua not so innocua

Listeria innocua is a Gram-positive, facultative, and rod shaped bacteria (Vazquez et al., 2001). This species is usually isolated from food samples and environmental sources, being well-known its ability to form biofilms (Allerberger, 2003). The typical single-species biofilm is formed by bacterial cells embedded in a self-produced matrix of extracellular polymeric substance in a wide range of pH and temperatures (Perni, 2005).Moreover, the biofilm formation is a currently problem in food processing factories, despite being considered a non-pathogenic bacterium (Allerberger, 2003; Slaghuis et al., 2004; Zapico et al., 1999). This environmental bacterium usually contaminates food throughout the production chain that goes from the environment, farm, slaughterhouse, processing plants and food products (Piginka-Vjaceslavova et al., 2020). In particular, *L. innocua* has been isolated in bovine meat, dairy, poultry, and seafood products (El-Shenawy, 1998; Kaszoni-Rückerl et al., 2020; Yehia et al., 2016).

Although *L. innocua* is considered a non-hemolytic, non-pathogenic bacterium, several studies reported atypical hemolytic strains containing virulence genes of *Listeria monocytogenes* such as *Listeria* pathogenicity islands, (Moura, Disson, & Lavina, 2019)and others virulence genes like *inlC, hly, plcA*, and *hly* (Moreno et al., 2014; Rossi et al., 2020). These genes are essential for intracellular infection, and thus the pathogenic potential of these *L. innocua* strains in human cells must be reconsidered. The importance of the present mini review is to describe the potential menace of the virulence findings in *Listeria innocua* on food safety and public health.

Mechanisms of pathogenicity in *Listeria innocua*

In the last years, the first reports of atypical strains of *Listeria innocua* with slight hemolytic activity had been report (Johnson et al., 2004; S R Milillo et al., 2012; Patocka et al., 1979; Volokhov et al., 2007) and subsequent studies showed the presence of LIPI-1 in these *L. innocua* strains (Johnson et al., 2004; Moura, Disson, Lavina, et al., 2019). In 2004, Johnson et al. demonstrated the presence of LIPI-1 genes and at least three active genes (*plcA, prfA, hly*) on an atypical *L. innocua* strain. In *Listeria monoyctogenes,* the *prfA* is a positive regulatory factor that controls the transcription of the genes of LIPI-1 island, while *plcA* encodes phosphatidylinositol specific phospholipase C that is involved in lysis of vacuoles, and *hly* encodes for listeriolysin O, which is a pore-forming hemolysin involved in phagolysosomal membrane lysis (Renzoni et al., 1997). Meanwhile, in 2019, Moura et al. demonstrated the presence of all LIPI-1 genes, including *mpl, pclB* and *actA*, that encodes a surface protein necessary for the polymerization of host cell actin, in atypical *L. innocua* isolates with hemolytic activity. However, it has been reported a clade, named FSLJ1-023, possessing a truncated *mpl* gene, being a gene responsible for the expression of a metalloprotein necessary for the maturation of the lectinase. Lectinase, encoded by the *plcB* gene, is necessary for vacuolar escape, therefore allowing the strains of this clade to establish a small infection foci and protrusions into vacuoles (Alvarez & Agaisse, 2016; Moura, Disson, Lavina, et al., 2019; Renzoni et al., 1997).

Nonetheless, the genes of LIPI-1 pathogenicity island are not the only virulence genes detected in *L. innocua*. Additional reports demonstrated the presence of *inlA* gene, encoding the leucine-rich repeat domain internalin protein InlA (Volokhov et al., 2007). Protein InlA is usually associated in bacterial invasion of non-phagocytic cells, as previously reported in *Listeria monocytogenes*. Also, the introduction of this gene in *L. innocua*, through *in vitro* assays, demonstrated its ability to infect mammalian cells (Gaillard et al., 1991). More recently,

it had already been reported an atypical strain with the capacity to infect cells naturally (Volokhov et al., 2007).

Additionally, other virulence factors were reported in certain *L. innocua* strains (Moura, Disson, Lavina, et al., 2019), including the functional pathogenicity island LIPI-3. This pathogenicity island is commonly found in *L. monocytogenes* linage I (serotypes 4b and 1/2b) and encodes a second hemolysin, known as listeriolysin S (LLS) (Cotter et al., 2008). LLS is only expressed in the presence of oxidative stress, allowing bacterial survival in polymorphonuclear cells (Cotter et al., 2008). In 2014, Clayton and colleagues evidenced the presence of LIPI-3 completed in *L. innocua* strains, where the natural inducible promoter (P_{llsA}) was replaced by a constitutive promoter (P_{HELP}). The authors proved the functionality of LIPI-3 island and also showed a hemolytic activity among the evaluated *L. innocua* isolates. These results supported several previous reports of fatal clinical cases from bacteremia and meningitis by certain strains of *L. innocua* (Clayton et al., 2014; Favaro et al., 2014; Perrin et al., 2003). Even though only isolated cases by *L. innocua* have been reported, it is important to mention the difficulties on the identification methods usually applied in diagnostic laboratories. The standard identification methods lack the specificity to differentiate between *L. monocytogenes* and *L. innocua* and atypical *L. innocua* strains. This methodological flaw complicates the differentiation of the causative agent of several infections with *Listeria* species. To solve this problem, a biochemical identification method has been proposed through Differentiation/Innocua/Monocytogenes (DIM) colorimetric tests for naphthylamidase activity, allowing to differentiate phenotypes of *Listeria* species through hemolysis (*Hly*), acid production from L-rhamnose (*Rha*) and D-xylose (*Xyl*) (FDA, 2018). DIM analysis nominally categorizes an isolate with the Hly^{+}, Rha^{-}, Xyl^{-} phenotype as a hemolytic *L. innocua* strain (see Table 1), if the strain is negative for *L. monocytogenes*-specific r-RNA by the AccuProbe or Gene-Trak tests. While *L. monocytogenes* is commonly identified as Hly^{+}, Rha^{+}, Xyl^{-}

phenotype (FDA, 2018). However, this methodology is a long and laborious process. Currently, it has been proposed a multiplex PCR procedure for the identification of *L. monocytogenes, L. innocua,* atypical *L. innocua* and *L. ivanovii* (Rosimin et al., 2016).

Recently studies characterized the virulence and infectivity of atypical strains of *L. innocua*, showing strains with LIPI-1 and a partial LIPI-3 pathogenicity island. These virulence factors demonstrated the ability of atypical *L. innocua* strains to cross the intestinal barrier and to spread into liver and spleen of zebrafish and murine models (Moura, Disson, Lavina, et al., 2019). In both *in vivo* models, the infective capacity was evaluated by oral and intravenous routes, demonstrating an intestinal invasion into 2 days of infection and a subsequent multiorgan dissemination (Moura, Disson, Lavina, et al., 2019). However, its infective capacity is lower than that reported for *Listeria monocytogenes*. In both *Listeria* species, the susceptibility to infect depends on the immune status of the host (Moura, Disson, Lavina, et al., 2019). Regarding to LIPI-3 pathogenicity islands, some *L. innocua* strains evidenced multiple mutations, such as *llsBYDP* genes that are needed to encode the LLS. The multiple mutations could unable these strains to invade and establish an infection in the host, when compared to *L. innocua strains* with the LIPI-3 island completed or without mutations (Clayton et al., 2014).

Furthermore, Moura and colleagues also found genes of the LIPI-4 pathogenicity island in *L. innocua* strains (such as, LM9005581_70009, LM9005581_70010, LM9005581_70011, LM9005581_70012, LM9005581_70013, and LM9005581_70014). These genes are probably related to carbon metabolism in saprophytic environments (Moura, Disson, Lavina, et al., 2019). However, the presence of LIPI-4 genes in *L. monocytogenes* serotype 4b is also associated with a more virulent phenotype, due to six genes involved in the expression of a sugar transporter system associated with neurological and placental tropism in the host (Maury et al., 2016). It is important to mention that *L. monocytogenes* serotype 4b is the most

phenotypically strain related to hemolytic serotypes of *L. innocua* (Maury et al., 2016). Finally, the presence of multiple genes from the LIPI-3, LIPI-4 and LIPI-1 islands have also been associated with certain pathogenic *L. innocua* strains (see Figure 1) (Radoshevich & Cossart, 2018; Yin et al., 2019), and therefore, further genomic studies should be carried out with these hemolytic strains of *Listeria innocua*.

Biofilm formation by *Listeria innocua*

Nowadays, the eradication of biofilms and their cross-contaminations represent the greatest challenges in the food industry (Galié et al., 2018). The ability to form biofilm is a mechanism that allows pathogenic bacteria to survive for long periods of time on biotic and abiotic surfaces (Grigore-Gurgu et al., 2019). For the *Listeria* genus, this ability has also been an advantage, allowing them to survive for long-term periods in adverse conditions of temperature and nutrient deprivation. The biofilm formation also allows an increase of antimicrobial resistance against biocides, such as, sanitizers (Skowron et al., 2019). In 2018, Luque-Sastre and colleagues compared the susceptibility between planktonic and biofilm cells of *Listeria* species against disinfectant treatments, showing the biofilms increased resistance against biocides, including *Listeria innocua*(Luque-Sastre et al., 2018). This augmentation of antimicrobial resistance in biofilms was observed in all biocides, such as 70% ethanol, benzalkonium chloride and the sanitizers P3-Alcodes, P3-Manodes, and Triquart MS. Only 48-hour biofilms were sensitive to triclosan biocide (Luque-Sastre et al., 2018)

Listeria innocua biofilms have been widely described in the food industry, not only due to cross contamination, but also as an indicator of contamination by *L. monocytogenes* (Aguado et al., 2004). The main reasons to use *L. innocua* as indicator of co-contamination with *L. monocytogenes* are the well-known association between them, forming multiple-species biofilms with other bacteria and allowing greater protection, perpetuation, exchange of

nutrients and transfer of genetic material (Sara R. Milillo et al., 2012). Food contamination by biofilms can occur in several steps during the product manufacturing (Awaisheh, 2010; Hoelzer et al., 2011; Lin et al., 2006), such as reception, production and packaging. These multiple contamination risks reduce product viability and storage, increasing a potential outcome of listeriosis among the population.

The phenotypic swift within the biofilm also leads to eradication challenges in food industry (Šilhová-Hrušková et al., 2015). Costa et al. evidenced a greater capacity of *L. innocua* in biofilm formation on dairy processing plants due to a more electronegative membrane and hydrophilic capacity (Costa et al., 2018). These properties allowed a better initial and irreversible adhesion to a surface increasing so biofilm formation. Therefore, the authors hypothesized that detection of false negatives of *L. monocytogenes* could frequently succeed among routine examinations. This possibility demands for a more precise identification of *Listeria* species and their serotypes in food processing plants, as previously stated in several studies (Carvalheira et al., 2010; Cornu et al., 2002; Kim et al., 2014; Petran & Swanson, 1993; Scotter et al., 2001).

In the dairy industry, contamination by *Listeria* species represents great losses in food supply, production of costs and manufacturing fees. In South Africa, 15 million dollars were reported in losses in export products in 2017 (Olanya et al., 2019). While a Belgian food company reported 30 million euros in product losses in 2018 due to contamination of fresh frozen vegetables. (Peeters, 2018). In terms of listeriosis costs in the United States, losses of 2.6 billion dollars per year were reported (Hoffmann et al., 2012) Meanwhile, in South Africa, losses of 10.4 million dollars were related to listeriosis cases (Olanya et al., 2019). In 2008, Canada reported losses of 242 million Canadian dollars (CA$) during an outbreak due to *Listeria monocytogenes* (Thomas et al., 2015). More recently, Skowron et al. (2019) analyzed a set of 250 cheese samples, detecting 2.8% of contaminated products by *L. innocua*. The

contamination by *L. innocua* was mainly found in cheese products, in particular Rockefort cheese (Skowron et al., 2019). Based on these results, the conditions of cheese industry manufacturing were simulated in the biofilm formation of *L. innocua* on stainless steel at different temperatures. Skowron and colleagues demonstrated *Listeria innocua* ability to form viable biofilms in 62 days at 4 ºC, 39 days at 20 ºC and 15 days at 37 ºC. Therefore, *L. innocua* showed the ability to form biofilm with a long survival period at different temperatures, correlating to its perpetuity in industrial contaminated surfaces and revealing an advantage to survive longer against adverse conditions (Skowron et al., 2019).

Low temperature is not the only adverse condition that *L. innocua* overcomes during biofilm formation. In 2019, Lezzoum-Atek et al. reported *L. innocua* ability to grow under microaerophilic conditions. Despite an initial slowdown in *L. innocua* growth, after 6 days, *L. innocua* biofilm was not different when compared to its growth in aerobic conditions. In addition, *L. innocua* also evidenced capacity to form multispecies biofilms, showing synergic and antagonist interactions based on the co-existing species in the initial biofilm formation. *L. innocua* revealed synergy interactions with *E. coli* in aerobic and microaerophilic conditions. On the other hand, antagonist interactions were observed in dual-species biofilms between certain strains of *L. innocua* and *L. monocytogenes*, revealing competition dynamics in the adhesion and biofilm formation steps (Lezzoum-Atek et al., 2019). These behaviors are already well-known in other multispecies biofilms (Burmølle et al., 2014; Elias & Banin, 2012; Liu et al., 2016), evidencing different social interactions among initial adherence and maturation during biofilm life cycle (Lezzoum-Atek et al., 2019). Further studies should be realized to identify synergistic, neutral, or even antagonistic effects among *L. innocua* and other foodborne pathogens.

Infectious cases induced by *Listeria innocua*

During the last two decades, infectious cases induced by *L. innocua* have been reported in both human and animal hosts (see Table 2). Even though *L. innocua* has historically been considered a non-pathogenic organism, several studies appointed *L. innocua* as a commensal bacterium and, in certain conditions, as an opportunistic pathogen being able to establish infection. In fact, early studies in murine models demonstrated the ability of *Listeria innocua* to induce listeriosis (Audurier et al., 1980; Lammerding et al., 1992). However, these cases of listeriosis were reported as lower extension of the infection, when compared to *L. monocytogenes*. In 2010, Bakker et al. demonstrated a hemolytic *L. innocua* strain with the ability to invade intestinal epithelial cells due to the presence of *inlA* gene. However, *in vivo* assays, the same strain was unable to invade cells of the murine models due to the absence of *inlC*, which it is necessary for invasion of mouse cells (Den Bakker et al., 2010). In 1994, the first case of meningoencephalitis caused by *L. innocua* was reported in a sheep, being accurately identified by API technique (Walker et al., 1994). Another infection caused by *L. innocua* was reported in an 18-month-old bµll, evidencing a neurological infection and leading to his death (P. Rocha et al., 2013). The autopsy revealed multiple lesions in the animal host, more exactly, multifocal microabscesses, vasculitis, edema and hemorrhage in the brain and cerebellum. Finally, *L. innocua* was identified from the mesencephalon biopsy and recognized as the causal agent of cerebral listeriosis, detecting also the presence of internalin *inlA* as an associated virulence factor (P. Rocha et al., 2013)

In 2003, a case of bacteremia by *L. innocua* with fatal offspring was reported for the first time in a human (Perrin et al., 2003). In this case report, a French 62-year-old female patient was hospitalized in with septic shock, progressing rapidly with neurological deterioration, acidosis, liver failure, disseminated intravascular coagulation, and multiorgan failure. The patient died within 40 hours of hospitalization. The analysis of the blood culture

revealed serotype 6a non-hemolytic *Listeria innocua* as the causal agent (Perrin et al., 2003). The following case report in human was reported by Favaro and colleagues in 2014. This case report was detected in an Italian patient with a chronic steroids' treatment. This immunocompromised patient appeared with typical clinical symptoms of meningitis (e.g., fever, nuchal stiffness, and impaired consciousness). A further microbiological analysis identified a non-hemolytic *L. innocua* serovar 4 as the causal agent. Several virulence factors associated with LIPI-1 genes were detected, such as *inlA* and *inlB* (truncated) genes (internalins) as well as *iap, clp* and *daaA* genes (genes usually found in pathogenic strains of *L. monocytogenes*). The antibiogram of this *L. innocua* serovar 4 revealed multidrug resistance against penicillin and oxacillin (Favaro et al., 2014).

Overall report cases reveal the need to fully characterize the potential of *L. innocua* as an opportunistic pathogen in both human and animal hosts on hemolytic *L. innocua* strains particularly.

Decontamination of *Listeria innocua* biofilms in the food manufacturing industry

The food industry faces serious challenges in the control of foodborne pathogens, such as *Listeria* spp., and it must not only to eradicate these pathogens but also their biofilms(Galié et al., 2018). During food manufacturing, sterilization processes should not affect the organoleptic properties of the products, needing to reinvent itself and seeking alternative technologies (Shah et al., 2019; Troy et al., 2016). Several strategies have been proposed for the eradication of *L. innocua* biofilms. Among them, the production of reactive oxygen species (ROS) is one alternative approach, affecting bacterial viability through direct damage to the membrane cells. ROS are able to increase the membrane permeability and, within the cell, to disturb cytoplasmatic proteins causing cell death (Kashef & Hamblin, 2017; Millan-Sango et al., 2015; Smet et al., 2016).

Recent studies reported the ROS production through different techniques, such as, photodynamic inactivation (PDI) (Xiong et al., 2019) and cold plasma inactivation (CAP) (Millan-Sango et al., 2015). PDI is based on the use of photostable porphyrins able to generate reactive oxygen species in the presence of visible light, allowing ROS formation directly in the processed food or any step in manufacturing process and storage (Hamblin, 2016). Commercially, porphyrins are usually obtained from on chitosan compounds (structural element in the exoskeleton of crustaceans) and applied to biofilm eradication (Castro et al., 2017). Chitosan is a biodegradable polymer with a well-known antimicrobial effect (against both fungal and bacterial pathogens), which has been showed to inhibit biofilm formation (Zhang et al., 2013). Although this technique reduces cell adhesion in biofilm formation, it is less effective on mature biofilms, especially during dark manufacturing conditions. Therefore, further characterization is still needed about porphyrins and their potential antibiofilm activity (Castro et al., 2017).

On the other hand, CAP has been evaluated on different biofilm and surfaces (materials), appointing to a greater antimicrobial effect on mature and full-developed biofilms. However, on porous material, authors reported that its effectiveness decreases (Xie et al., 2019). Due to this technical disadvantage, it has been tried to combine CAP technique with other antimicrobial treatments, such as nisin, a natural compound obtained from *Lactococcus lactis* with antimicrobe activity. This compound induces its antimicrobial activity due to its union with lipids required for the formation of membranes in Gram positive bacteria. Although it was found highly effective against planktonic cells, its effectiveness decreases in biofilms due to the amount of extracellular matrix (EPS). Also, the order of the combined treatment was reported to be equally important, being necessary first to apply the nisin and then the CAP (Costello et al., 2021).

Other natural antimicrobial compounds have also been evaluated on foodborne pathogens, such as bacteriocins produced by enterococci (e.g., *Enterococcus faecium* and *Enterococcus faecalis*) (Al-Seraih et al., 2017; Gómez et al., 2012). Using the cell-free supernatant of enterococci broth culture, authors evaluated the antimicrobial effect of this biosurfactant (bacteriocins) against *Listeria innocua, Listeria monocytogenes* and *Listeria ivanovii* biofilms. The results demonstrated a greater antimicrobial activity in *L. innocua* biofilms, when compared to the remaining *Listeria* species. Moreover, the antimicrobial activity of this biosurfactant evidenced a greater decrease in both biomass and cell viability of full-developed and initial biofilms, respectively. Although this biosurfactant showed promising results, its efficiency is still variable depending on certain factors, such as temperature (optimal effect at 37ºC). Further studies are needed to fully characterize the antibiofilm effect of this biosurfactant (bacteriocins) (K. R. Rocha et al., 2019).

Other important factor to eradicate *L. innocua* biofilms on surfaces and food products are multispecies biofilms (Behnke et al., 2011; Millezi et al., 2012). As well-known, multispecies biofilms showed greater resistance to disinfectants, when compared to monospecies biofilms, illustrating a more complex quorum sensing, EPS composition and biofilm structure (Kocot & Olszewska, 2020) . This complexity was evidently demonstrated in a study realized by Kocot and Olszewba in 2020. These authors evaluated the antimicrobial effect of three types of disinfectants on dual-species biofilms, more exactly, biofilms formed by *L. innocua* with *S. aureus* and *L. innocua* with *P. aeruginosa*. These disinfectants were based on tertiary alkyl amine, chlorine, and quaternary ammonium compounds (QACs). In both cases, dual-species biofilms evidenced a greater resistance to every disinfectant, when compared to monospecies biofilms. However, the authors reported different behaviors of these dual-species biofilms against each disinfectant. For example, the *L. innocua-S. aureus* biofilm showed more resistance against chlorine-based disinfectant and in full-developed biofilms. On

the other hand, *L. innocua-P. aeruginosa* evidenced a higher sensitivity in full-developed biofilms, when compared to initial biofilms, and no particular resistance was observed among the evaluated disinfectants (Kocot & Olszewska, 2020).

One alternative approach from standard disinfectants could be other commercial sanitizers, such as Micro E-pro. This latest product is constituted by flavonoids (such as naringenin), glycerin, lactic acid, and citric acid. Originally this combination was obtained from concentrated bitter orange extract. In 2020, Medina-Rodriguez and colleagues also evaluated Micro E-pro effectiveness against a dual-species biofilm formed by *L. innocua* and *Pseudomonas* spp. This study was found an antimicrobial effect of Micro E-pro comparable to sodium hypochlorite disinfectant, but it also had the advantage of being natural and biodegradable without affecting the properties of food product (Medina-Rodríguez et al., 2020). However, when evaluating Micro E-pro against other species, the antimicrobial effect was obviously dose dependent and, at similar doses, the product effectiveness varied among different species (Medina-Rodríguez et al., 2020).

These studies revealed the importance of knowing the dynamic and the behavior of biofilm consortia to establish the best disinfection strategies and to search for alternative products for biofilm control (Fancello et al., 2020; Wang et al., 2020). Nowadays, sterilization of manufacturing surfaces and eradication of *Listeria* species biofilms in products remain a difficult challenge for food industry. Further studies are needed to fully characterize the diversity and dynamic of foodborne pathogens to establish monospecies and multispecies biofilms, contributing for food safety and reducing millionaire losses of the manufacturing industries.

PART II – SCIENTIFIC ARTICLE

CHARACTERIZATION OF MONOSPECIES BIOFILM CYCLE LIFE BY *LISTERIA INNOCUA*

Introduction

The members of genus *Listeria* are present in the environment and its ability to colonize food processing plants and products is well-known (Shamloo et al., 2019). *Listeria monocytogenes* and *L. ivanovii* are common etiological agents of listeriosis and responsible for outbreaks around the world (Guillet et al., 2010; Tchatchouang et al., 2020). Fifteen *Listeria* species are usually classified as harmless environmental saprophytes (Rosenberg et al., 2014). However, several studies reported opportunistic infections in immunocompromised patients caused by *L. innocua* (Perrin et al., 2003), *L. seeligeri* (Rocourt et al., 1986), or *L. grayi* (Salimnia et al., 2010). *L. innocua* on food processing plants surfaces is usually coexisting with *L. monocytogenes* (Jeon et al., 2018). In 2019, Skowron et al. detected *L. innocua* on surfaces at different temperatures including freezer areas, on abiotic or biological surfaces (Skowron et al., 2019). Its ability to form biofilms and survive for a long time was initially showed by Korsak and Szuplewska in 2016 and then confirmed in another study done by Jeon et al. (Jeon et al., 2018; Korsak & Szuplewska, 2016).

However, little is still known about *L. innocua* cycle life in monospecies and multispecies biofilms. It is important to understand the dynamic of biofilm formation of *L. innocua* to find ways to control and prevent it in the manufacturing food industry. So, the main goal of this study was to characterize *L. innocua* monospecies biofilm formation at different temperatures and its biofilm cycle life at optimal temperature.

Materials and methods

Growth culture and biofilm assays

Listeria innocua Li148 strain was obtained from soft cheeses of a popular market in Cotopaxi and taken to the Microbiology Institute of the Universidad San Francisco de Quito (MI-USFQ) (Espinosa-Mata et al., 2020). This strain was isolated and pre-cultured in Tryptic Soy Agar (TSA) at 37°C for 18 hours. The culture was then diluted in a sterile falcon tube with Tryptic Soy Broth (TSB) to an initial OD 600 nm of 0.2 (to an equivalent concentration of 10^7 UFC/ml; see Standard Growth Curve in annex A), as previously performed by Costa and colleagues (Costa et al., 2018).

All biofilm assays were performed under static conditions on glass coverslips within 6-well plates; two wells were used as a negative control with 3 ml of sterile medium and the remaining ones as an assay using 3 ml of medium with bacterial inoculum.

The optimal temperature for biofilm formation was determined at 4, 22, and 37°C for 48 hours, and then biofilm formation was evaluated. After this initial evaluation, additional 6-well plates were incubated at 37°C for 12, 24, 48, 72, 80, and 96 hours. In each experimental assay, the TSB medium was replaced every 24h. All experimental assays were carried out in triplicate on different days.

Optical Density and Colony-forming units measurements in *L. innocua* biofilm samples

After incubation time was completed, TSB medium was carefully removed from each well, softly rinsed with sterile phosphate-buffered saline (PBS) on the lateral wall of the well (approximately 45º of angle). In selected wells, 1.5 ml of violet crystal at 3% in ethanol (96%; v/v) solution was added for two minutes. Then the solution of violet crystal was removed and 200 µl of acetic acid solution at 33% (v/v) was added in each well for 5 minutes, as performed in previous studies (Alonso et al., 2014; Costa et al., 2018). Finally, each well

was measured for biofilm formation at OD 630 nm through an Elx808 spectrophotometer (BioTek, Winooski, USA).

To evaluate biofilm formation through culture media, selected coverslips were placed in 3 ml of sterile PBS and then vortexed for two minutes. Finally, the obtained PBS solution was used in serial dilutions and cultured in TSA for 24h. Colony-forming units (CFU) were counted to estimate the number of viable bacteria cells in samples of each experimental condition, as standardized in previous studies (Heir et al., 2018; Jeon et al., 2018; Skowron et al., 2019).

After an experimental evaluation of the OD and CFU measurements in *L. innocua* biofilm, additional assays were carried out to evaluate biofilm growth through classical and fluorescence microscopy.

Analysis of biofilm growth by classical and fluorescence microscopy

For classical microscopic analysis, coverslips were submerged in a solution of violet crystal at 3% in ethanol (96%; v/v) for 5 minutes and washed with PBS three times. Coverslips were dried at room temperature and then analyzed with optical microscopy (Olympus, CX22LED, Tokyo, Japan), accordingly with previous studies (Costa et al., 2018; Machado et al., 2013; Merritt et al., 2011). Under 1000x magnification, pictures were taken from the center and the surrounding area of the coverslip using AmScope Digital Camera MU633-FL (AmScope, California, USA) and digitalized with AmScope software version 1.2.2.10.

For fluorescence microscopy analysis, samples of biofilm growth after 96 hours at 37°C were selected to evaluate matured biofilms. In these experiment assays, cell viability in biofilm structure was determined using a LIVE/DEAD® Cell Vitality Assay Kit (L34951, Invitrogen, probes.invitrogen.com/media/pis/mp34951.pdf, Invitrogen, Carlsbad, California, USA) following manufacturer's instructions. Under 1000x magnification, pictures of the coverslips

area were acquired with an Olympus BX50 microscope (Olympus Corporation, Tokyo, Japan) equipped with an AmScope Digital Camera MU633-FL and digitalized with AmScope software version 1.2.2.10.

Development of a quantitative Polymerase Chain Reaction (qPCR) standard curve

To create a quantification standard curve and positive controls, a solution with a known concentration of Listeria innocua (CFU / mL), a sample of known concentration (also known as a calibrator) (for example, number of CFUs per mL) was obtained over a validated calibration curve (CFU / OD)(Begot et al., 1996). Calibrator was serially diluted through a controlled series and used to construct a standard curve for qPCR assays. The DNA extraction was performed using PureLink Genomic DNA Mini Kit (Invitrogen, USA) by adding lyticase from *Arthrobacter luteus* (Sigma-Aldrich, German) from the highest CFU/ml concentration, and serial dilutions allowed us to have concentrations from 10^9 CFU/mL, which were used as qPCR standards. Primers used were previously described to amplify only *Listeria innocua* (Tao et al., 2017) through classical PCR, but not tested for quantitative real-time PCR and were *in*9F (GGCTTCAGCGATTCTTCCG) and in9R (GCCCGATTTCCTCACTGTCTAA) with a PCR product size of 421 bp. The reaction contained 5 µl of GoTaq qPCR Master Mix (Promega, Madison, WI, USA), 0.5 µl of primers in 9F, 0.5 µl of primers in 9R, 3 µl of H2O and 1 µl of DNA. CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad, California USA) was used with denaturation for 5 minutes at 94°C followed by 35 cycles of 30 s at 94°C of denaturation, 30 s at 58°C of annealing and 60 s at 72°C of extension and final extension for 10 minutes at 72°C. The standard measurement of SYBR Green fluorescence was performed during the elongation phase at 72°C above the temperature of melting (Tm) of the primers. The melting curve analysis from 65°C to 95°C with an increase of one degree Celsius every 30 s. Each qPCR assay was followed by this melt curve analysis. The range of linearity

of the assay was from 10^9 to 10^4 CFU/mL. *Listeria innocua* load in each sample was measured by running six or five standard solutions (10^9-10^3 CFU/mL), both in duplicate or triplicate on each qPCR assay. The qPCR results were considered negative if the cycle threshold (CT) values were superior to 35. Each assay was analyzed by triplicate, and two no template controls were included as negative controls (only qPCR reagents without *Listeria innocua* DNA; also known as no template controls).

Statistical analysis

Statistical analysis was performed using the statistical software JASP (JASP version 0.13, http://www.jasp-stats.org, JASP, Amsterdam, The Netherlands)[8]. *L. innocua* growth was analyzed through One-way ANOVA (ANalysis of Variance) with posthoc Tukey HSD (Honestly Significant Difference) test calculator for comparing multiple experimental conditions (different temperatures and hours in Biofilm formation). The results were considered statistically significant at $P \leq 0.05$.

Results

Initial evaluation of *L. innocua* biofilm growth on different temperatures

Initial assays demonstrated the ability of *L. innocua* to produce viable biofilm after 48 hours of incubation on the glass surface at 4, 22, and 37 °C, as shown in Fig. 2A. The biofilm growth was significantly incremented at 37°C (P <0.001; 1.30E09 CFU/ml ± 4.65E06) in comparison with biofilm growth at 22 °C (7.0E08 CFU/ml ± 5E07) and 4°C (8.53E07 CFU/ml ± 1E08). The lowest levels of biofilm growth were observed at 4°C. Concerning biofilm mass, our results showed similar trends as previously obtained by CFU counting. Biofilm mass was statistically augmented at 37 °C (P <0.001; OD630: 0.274 ± 0.01) when compared to the

remaining temperatures of biofilm growth (see Fig. 2A). The lowest biofilm mass was also detected at 4°C (0.045 ± 0.00).

As shown in Fig. 2 B – D, optical microscopy analysis was done to differentiate biofilm stages of *L. innocua* on the glass surface at different temperatures. At 4°C, *L. innocua* only produced a rudimentary biofilm (Fig. 2B). While, at 22°C, *L. innocua* was able to establish an initial biofilm covering almost all glass surface (Fig. 2C). Finally, at 37°C, *L. innocua* formed a full biofilm with a complex three-dimensional structure covering the complete area of the glass surface (Fig. 2D).

Longitudinal analysis of *L. innocua* biofilm growth

After an initial evaluation for biofilm growth, the *L. innocua* ability to establish a biofilm development at 37°C across the time was evaluated at 12, 24, 48, 80, and 96 hours (see Fig. 3). As expected in biofilm cycle life, *L. innocua* produced the lowest biofilm at 12 hours (1.64E+0.8 ± 3.83E+07) showing a statistically significant lower viable cell counting to other biofilm timelines at 12 and 24 hours (P <0.001). The peak of *L. innocua* biofilm growth was observed at 72 hours (1.70 E+0.9 CFU/ml ± 1.24E+08), followed by a continuous reduction at 80 hours (1.59E+09 CFU/ml ± 8.98E+07) and 96 hours (1.37E+09 CFU/ml ± 2.04E0.8). This decrease of biofilm viable cells showed a statistically significant difference when comparing to the peak hour (P < 0.05; see Fig. 3A).

When analyzing the evolution of biofilm mass, *L. innocua* showed a continuous augmentation across time. As expected, the lowest biofilm mass was at 12 hours (0.035 ± 0.01) increasing by 322.34% at 24 hours (0.070 ±0.00), 63.85% at 48 hours and 49.85% at 72 hours (OD630: 0.750 ± 0.019). All these biofilm timelines showed statistically significant differences against 48h of biofilm growth (P <0.001). Finally, the higher measure of biofilm mass was determined at 96 hours (0.797 ±0.04; see Fig. 3A).

Optical microscopy analysis validated the previous results on biofilm mass at different biofilm cycle life stages (see Fig 3B – D). After 12 hours, *L. innocua* established an irreversible initial adhesion on the glass surface showing sparse aggregates of cells (Fig 3B). At 24 hours, *L. innocua* was able to achieve a soft layer (initial biofilm stage) covering almost the glass surface (Fig. 3C). Then, at 48 hours, *L. innocua* biofilm was observed as a strong layer covering the complete area of the glass surface (mature biofilm; see Fig. 3D). However, after 48 hours, all images of *L. innocua* biofilm in different timelines were saturated, and so it was unfeasible to differentiate the biofilm evolution.

However, live/dead assay with fluorescence microscopy analysis was carried out at 96 hours timeline to evaluate cell viability in a full mature biofilm of *L. innocua*. Our results showed a multilayered cellular structure, evidencing a regular distribution of viable (green) and dead (red) cells in the peripheric area of the analyzed biofilm (mean of 59.48% of live cells *versus* 40.51% of dead cells; see Fig. 3E). In the center area of the biofilm, it was possible to detect a major biofilm growth demonstrating the largest population of dead or stressed cells (mean of 46.13% of live cells *versus* 53.86% of dead cells; see Fig. 3F). However, no statistically significant differences were found between peripheric and center areas of the biofilm. Also, it is important to mention that *L. innocua* biofilm at 96 hours was mainly formed by live cells in the present analysis.

Evaluation of a quantitative Polymerase Chain Reaction standard curve for *L. innocua*

The quantitative PCR calibration curve showed a positive correlation between all points with an R2 of 0.999. The differences between each point are stable between 3 to 4 cycles of difference as the dilution increases (Fig. 4). The negative controls did not show any amplification curve during the experimental assay, indicating no internal contamination of the reagents or media. Finally, the melting curve was uniform with a single peak at 79°C and no

other peaks that could indicate any cross-contamination in the experimental samples (Fig. 5), demonstrating a validated procedure development for future qPCR assays.

Discussion

Our results suggest that *L. innocua* can adhere and form biofilm on sterile glass surfaces regardless of temperature. This strain was able to produce biofilms under standardized conditions indicating a regulatory system that facilitates adaptation to different environmental conditions. However, the biofilm growth by *L. innocua* evidenced statistically differences according to temperature, similar to a previous study with other *Listeria* spp. reporting the enhanced biofilm growth with increased temperature(Bonsaglia et al., 2014; Colagiorgi et al., 2017). Other studies also reported a rudimentary biofilm at 4°C and reported 37°C as the optimum growth temperature (Fan et al., 2020; Kocot & Olszewska, 2017). This superior biofilm growth at higher temperatures could be partially attributed to the augmentation of metabolic pathways and hydrophobicity of cell surface allowing the adhesion of bacterial cells to an abiotic surface (Fan et al., 2020). In multispecies biofilm between *L. monocytogenes* and *Salmonella enterica* subsp. *enterica* serotype Typhimurium (usually abbreviated to *Salmonella* Typhimurium) has already been reported (Govaert et al., 2019). These authors showed a different optimum growth temperature in dual-species biofilm formation, evidencing an optimal temperature lower than 25°C. In 2012, Kostaki et al. demonstrated that optimum conditions can change between single and multispecies biofilm (Kostaki et al., 2012). In this particular case, this dual-species biofilm showed a cooperative relationship where *Salmonella* Typhimurium provided nutrients to *L. monocytogenes* (Kostaki et al., 2012) but the biofilm growth strictly depended on *Salmonella* Typhimurium optimal environmental conditions (temperature).

In our study the initial biofilm of *L. innocua* was first observed after 12 hours, achieving the biofilm growth peak at 72 hours, after 72 hours, the biomass increased but the cell viability decreased at 96 hours. These results agreed with a previous thesis report done by Berrón Jiménez (Berron & Rodriguez, 2016), where a maximum growth peak of the *L. innocua* biofilm was reported at 72 hours. Other studies reported different life cycles depending on strains' ability to form biofilms in *L. monocytogenes* (Kadam et al., 2013; Lezzoum-Atek et al., 2019). *L. monocytogenes* strains with the highest biofilm formation capacity showed the maximum peak at 36 hours while strains with weak biofilm showed a slower formation rate and a quick dispersion after 60 hours (Fan et al., 2020).

These differences in biofilm formation capacity could be due to intrinsic properties, such as hydrophobicity (more hydrophobic properties formed stronger biofilms) and motility. So, properties, other than medium culture and temperature, modulated biofilm formation. It has been reported that the *CheY* gene is involved with both motility and hydrophobicity in *L. monocytogenes* and its biofilm formation capacity since mutant strains possessed a lower biofilm formation (Fan et al., 2020). Although this gene has also been found in *L. innocua* (Glaser et al., 2015), its role in *L. innocua* biofilms needs to be further studied. Moreover, Kostaki et al. (2012) and Govaert et al. (2019) also reported a fast-mature biofilm development in their dual-species biofilm assays (*L. monocytogenes* and *Salmonella* Typhimurium), showing a biofilm growth peak after 10 hours in optimal conditions and a biomass augmentation until 18 hours(Govaert et al., 2019; Kostaki et al., 2012). Therefore, single and multispecies biofilms showed a different evolution due to antagonism and synergic interactions. Further studies must be performed to fully characterize these dynamic interactions. An important point to take into account in the counting of colony-forming units is that there are bacterial groups in the biofilm that are persistent, these are cells that are metabolically inactive and therefore do not have activity or grow. It is necessary to

take into account this cell subgroup because due to their low metabolic activity they become more resistant to antibiotics and disinfectants; however, this resistance is due to its inactivity and not to mutations or genetic changes. Within the biofilm this subpopulation is important since it has been associated with the persistence of chronic infections resistant to antibiotics(Wood et al., 2013).

Microscopic analysis of *L. innocua* mature biofilms showed a honeycomb-like structure with hollows of different sizes suggesting a cell death dispersion. This patron was already reported in other studies as advantageous for biofilm development of *L. monocytogenes* due to the improvement of the biofilm stability and higher absorption of nutrients by the consortium (Reis-Teixeira et al., 2017; Ripolles-Avila et al., 2018). The percentage of cell viability on *L. innocua* biofilms in our study oscillated between 46.13% to 59.48% at 96 hours, similar to a previous study for *L. monocytogenes* biofilm (Ripolles-Avila et al., 2018). *L. monocytogenes* biofilms at 96 hours also illustrated similar groups of adhered bacteria to the abiotic surface (Kocot & Olszewska, 2017; Reis-Teixeira et al., 2017), as shown in *L. innocua* biofilms of the present study. To authors best knowledge, this is the first study to report the biofilm architecture and viability range of the biofilm cycle life by *L. innocua* until 96 hours of culture.

In summary, this study confirmed the ability of *L. innocua* to form biofilms at different temperatures and characterized its cycle life and architecture structure. The results also revealed a similar behavior previously reported in *L. monocytogenes* biofilms, appointing *L. innocua* as a good *in vitro* model and indicator of *L. monocytogenes* biofilms in the food industry. Finally, the strong ability to form biofilms of *L. innocua* in the food industry should be considered as a public health issue due to recent reports of some virulent hemolytic strains.

Conclusions

L. innocua was able to form biofilms at any temperature tested, achieving its maximum growth at 37°C. Biofilm production increased for 72 hours until reaching a stationary and mature biofilm. In the stationary phase, the colony-forming unit (CFU) counting of the biofilm diminished at 96 hours while microscopy analysis and crystal violet measurement showed an increment of biomass. Live/dead assays showed a survival rate of 56.67% (±3.97). Our results demonstrated a great adaptation of *L. innocua* biofilms to thermal conditions and showed a full life cycle with 96 hours of biofilm growth. At 72 hours, *L. innocua* achieved the growth peak revealing a mature biofilm until 96 hours. Then *L. innocua* biofilm began to lose cell viability in their biomass, and probably initiated the dispersion phase of the life cycle. It is important to mention some limitations of the present study, such as, no quantitative PCR was realized to validate the total number of *L. innocua* cells (viable and no viable cells) and no confocal analysis to evaluate the distribution of viable and non-viable cells on *L. innocua* monospecies biofilm during time. Additionally, the count of colony forming units (CFU) in the biofilm has limitations when counting viable but non-culturable cells, so it needs to be taken into account. Future studies should be done with multispecies biofilm of *L. innocua* to establish synergic and antagonist relationships between foodborne pathogens also found in manufacturing production and characterize the biofilm-forming ability of the atypical hemolytic strains of *L. innocua*.

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TABLES

Table 1. Main phenotype characteristics detected in Listeria innocua isolated of reported infectious cases.

L. innocua **strain**	***Hly***	***Rha***	***Xyl***	**Biotype**
Non-hemolytic strain	-	+	-	2,3,5 (common strains)
Non-hemolytic strain	-	-	-	2,3,5 (common strains)
Hemolytic strain	+	-	-	4,7(atypical strains)
Hemolytic strain	+	+	-	Non reported (atypical strains)

The table represents the main biochemical parameters established for the differentiation of different strains of *Listeria innocua*. *Hly* + correspondence to the strains can produce hemolysis due to the presence of listeriolysin O. Rha + corresponds to the strains capable of producing acid from rhamnose, this is given by the inducible promoter *P rha*. *Xyl* + correspondence to the strains can ferment the D-xylose, this strains can product acid from D-xylose carbohydrate(FDA, 2018; Fieseler et al., 2012; Orsi & Wiedmann, 2016)

Table 2. Summary of* Listeria innocua *reported cases on humans and animals.

Case	*L. innocua* strain	Virulence factors	Country	Prognostic	Host	Year	References
Bacteremia in a 62-year-old woman.	Serovar 6a	No reported	France	Dead	Human	2003	(Perrin et al., 2003)
Meningitis in a 72-year-old immunocompromised patient (on steroids and etanercept).	Serovar 4	Listeriolysin O, LIPI-1 pathogenic island (except genes *actA* and *pclB*). Internalins (*inlA, inlB*)	Italy	Dead after 10 days	Human	2014	(Favaro et al., 2014)
Meningoencephalitis in ovine	Non reported	Absence of *prfA* and *hly* genes	United Kingdom	Sacrificed after 24 hours	Ovine	1994	(Walker et al., 1994)
Cerebral listeriosis in an 18-month-old Blonde Aquitaine bµll	Non reported	Internalin *inlA*	Italy	Died after 24 hours	Ruminant	2012	(P. Rocha et al., 2013)
Multidrug-Resistant *Listeria innocua* Strain Isolated from a Wild Black Bear feces	Non reported	*tet*(M), *ant*(6)-Ia_2, and *mphB* resistant genes for tetracycline, aminoglycosides, and macrolides.	Egypt	Non reported	Bear	2019	(Uam-a et al., 2019)
Non-purµlent periventricularly localized encephalitis in suckling mice	Non reported	Non reported	Germany	Sacrificed	Mice	1976	(Patocka et al., 1979)
Three cases of aborted calves. A fetus with bronchopneumonia, enteritis and DIC. Two fetuses with agonal changes and acute hypoxia.	Non reported	Non reported	Latvia	Aborted	Calves	2017-2019	(Piginka-Vjaceslavova et al., 2020)

FIGURES

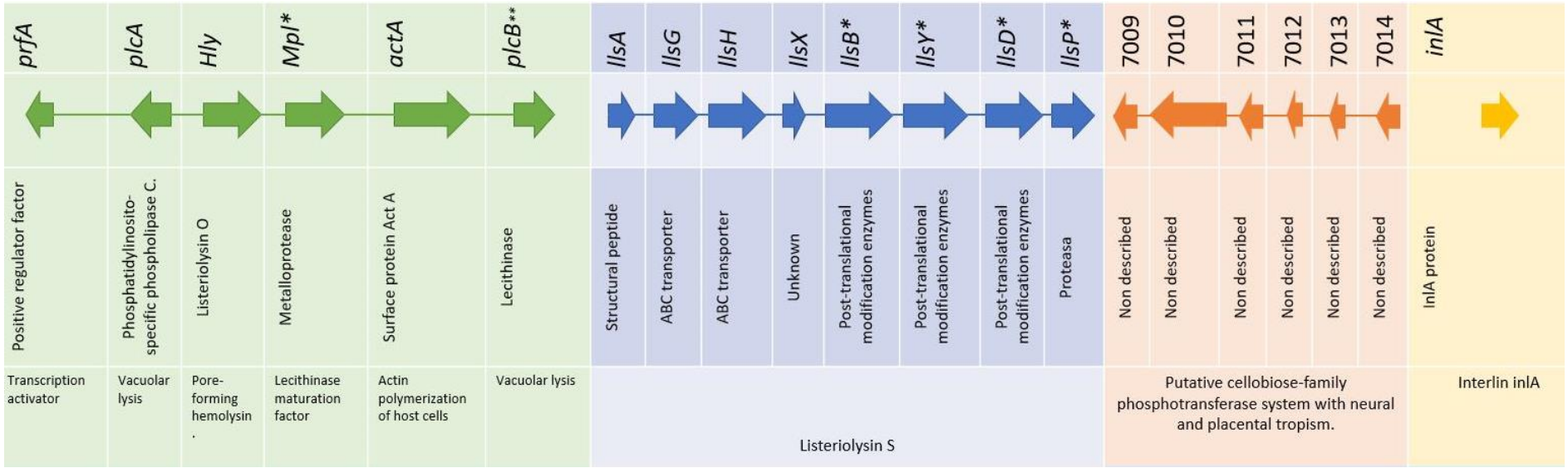

***Fig. 1. Islands of pathogenicity and main virulence factors associated with* L. innocua.**

The LIPI-1 pathogenicity island includes genes that encode for virulence factors, such as *hly* that encodes for Listeriolysin O; *actA* encoding an actin polymerization protein, *pclA* and *pclB* encoding a phospholipase C and lecithinase respectively; all this controlled by *PrfA*, a central regulator of gene expression through the activation of promoters that have palindromic DNA sequences, the *PrfA* box. *PrfA* also controls the expression of *inlA*, which encodes internalin A, necessary for invasion into the host cell (Hadjilouka et al., 2018; Vazquez et al., 2001). The LIPI-3 pathogenicity island includes 8 genes involved in the synthesis of Listeriolysin S, which is a hemolysin involved in bacterial survival within nuclear polymorphs and is only transcribed under conditions of oxidative stress(Clayton et al., 2014)(Vilchis-Rangel et al., 2019). The recently described LIPI-4 pathogenicity island is a set of 6 genes LM9005581_70009 to LM9005581_70014) that codes for a sugar transport system (of the cellobiose family) and has been associated with an increased risk of severe infection by its neurological and placental tropism(Maury et al., 2016).

Legend: * May be absent or truncated. ** Dependent on *mpl* gene to activate, if *mpl* is inactive this too.

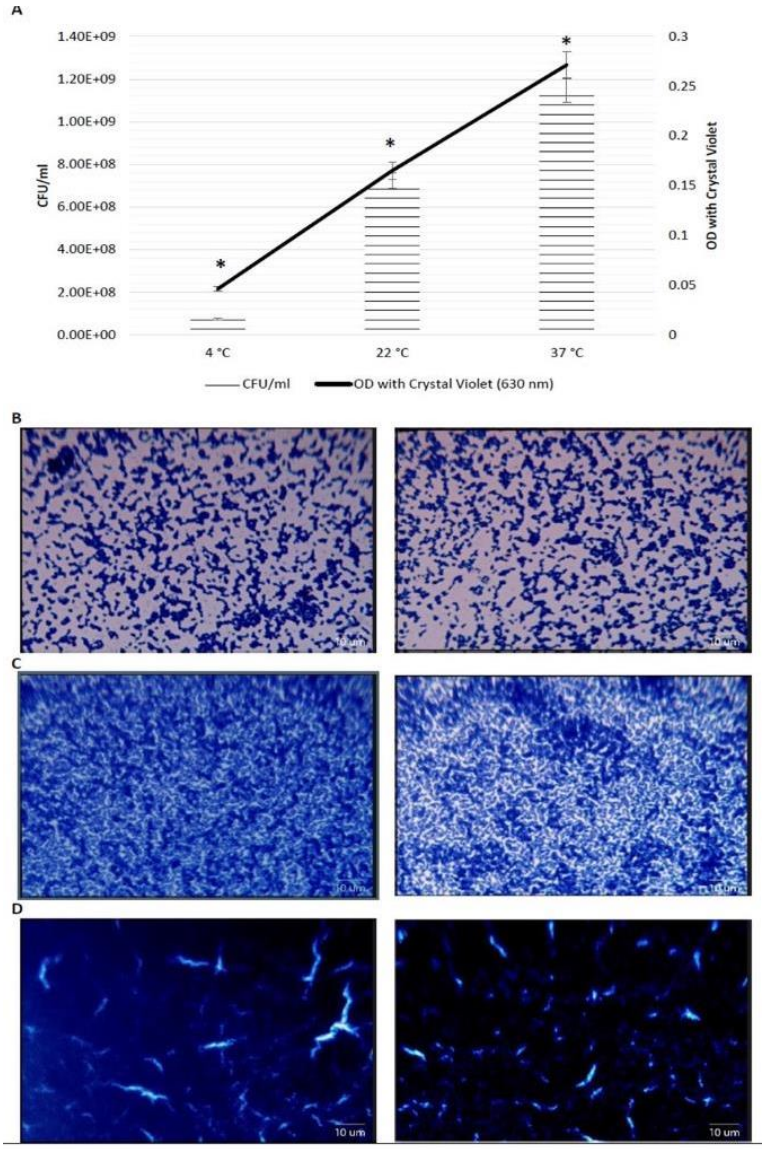

Fig. 2. Analysis of biofilm formation of L. innocua based on temperature.

A The bars represent the viable cells of *L. innocua* in monoculture biofilms after 48 hours of incubation at 4°C, 22°C and 37°C. The lines represent the OD at 630 nm with Crystal Violet of *L. innocua* in monoculture biofilms after 48 hours of incubation at 4°C, 22°C and 37°C. Each data point is the result of the mean of triplicate (bars represent the standard error of the mean) Standard deviation less than 10% for each point. * indicates statistical significance temperature with the highest amount of CFU/ml by Tukey test at 5% probability error with a P-value<0.001. **B** Photomicrograph (1000X) showing *L. innocua* biofilm at 4°C for 8 hours on a coverslip with 3% crystal violet. **C** Photomicrograph (1000X) showing *L. innocua* biofilm at 22°C for 8 hours on a coverslip with 3% crystal violet. **D** Photomicrograph (1000X) showing *L. innocua* biofilm at 37 °C for 8 hours on a coverslip with 3% crystal violet.

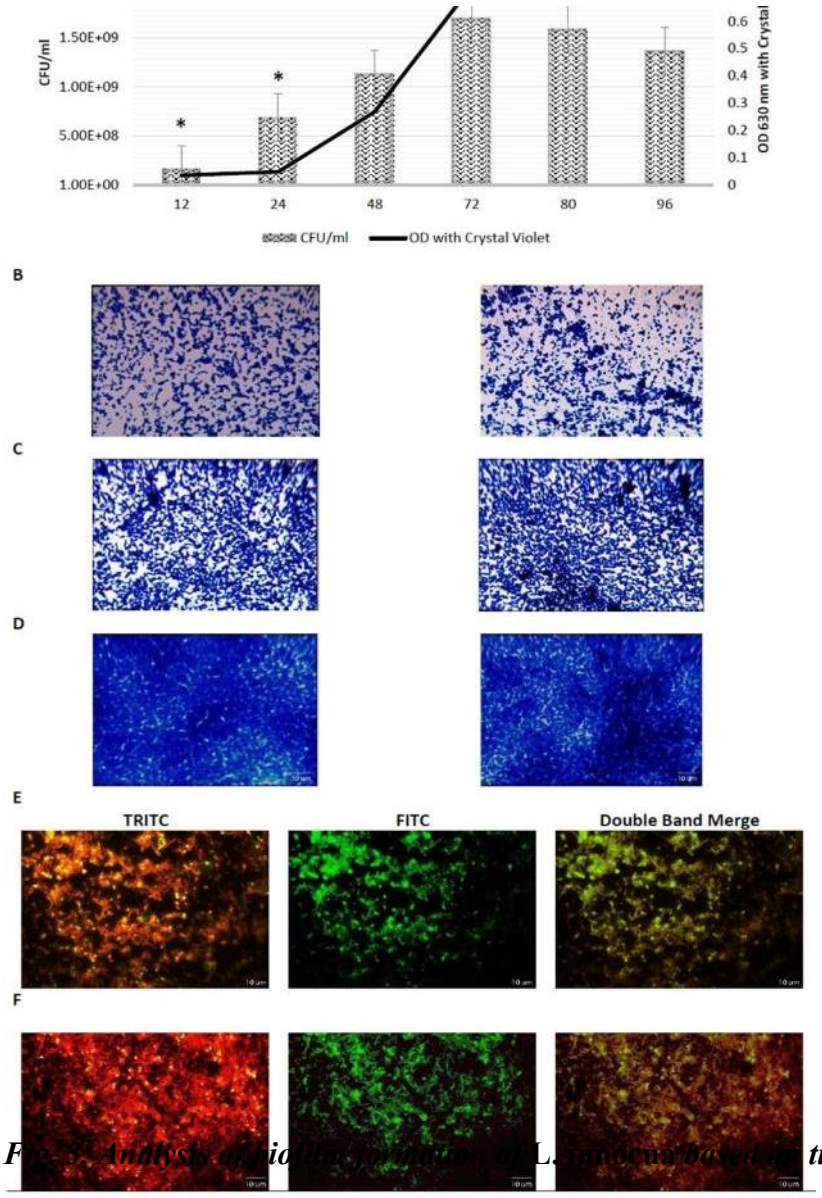

Fig. 3. Analysis of biofilm formation of L. innocua based on time.

A Bars represent viable cell counts at different hours at 37°C at 12, 24, 48, 72, 80 and 96 hours of *L. innocua* biofilm in sterile glass. The Line represents the OD with Crystal Violet at 630 nm at 37°C at 12, 24, 48, 72, 80 and 96 hours. Each data point is the result of the mean of triplicate (bars represent the standard error of the mean). * Indicates statistical significance by Tukey test at 5% probability error with P-value<0.001. **B** Photomicrograph (1000X) showing *L. innocua* biofilm at 37°C for 12 hours on coverslip with 3% crystal violet. **C** Photomicrograph (1000X) showing *L. innocua* biofilm at 37°C for 24 hours on coverslip with 3% crystal violet. **D** Photomicrograph (1000X) showing *L. innocua* biofilm at 37°C for 48 hours on coverslip with 3% crystal violet. **E** Photomicrograph (1000X) showing *L. innocua* biofilm at 37°C for 96 hours on coverslip with *Live/dead* kit. **f** Photomicrograph (1000X) showing *L. innocua* biofilm at 37°C for 96 hours on coverslip with *Live/dead* kit.

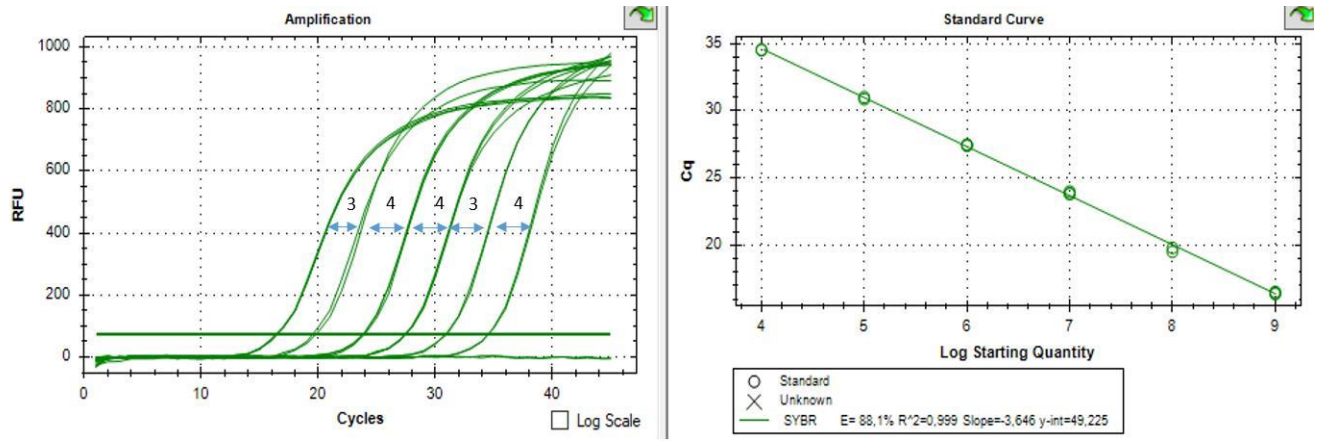

***Fig. 4. Amplification of standard points and Standard Curve to quantify* Listeria innocua.**

The image on the left shows the amplification curve for each point from 10 ^ 9 to 10 ^ 4. The arrows and the numbers show the number of cycles of difference between the points. The image on the right shows the standard curve of the points with R*2 = 0.999, the circles correspond to the values with known concentrations used for standardization.

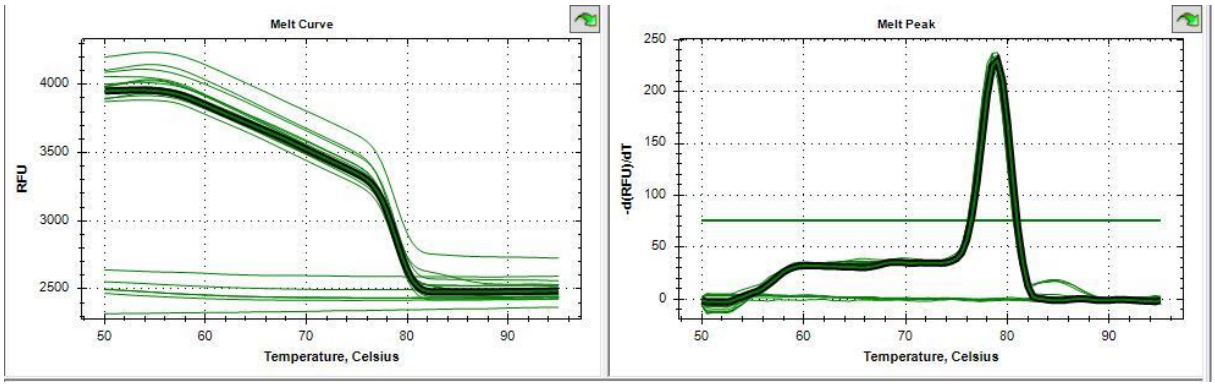

***Fig. 5. Profile of Melting Curve and the specific temperature of melting (T_M) to quantify* Listeria innocua.**

The image on the left corresponds to the melting curve, it can be seen that the negative controls had no amplification. The image on the right corresponds to the Melting Peak where it can be seen that all concentrations had a standard peak at 79°C.

ANNEXES INDEX

Annex A: Listeria Innocua Optical Density Calibration Curve…… 62

ANEEX A: **Listeria Innocua** *Optical Density Calibration Curve*

Assays were performed in triplicate on different days and the average of the points is shown at 600 nm.