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BIOFILM FORMATION OF *LISTERIA* SPECIES UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

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1. INTRODUCTION

Food-borne diseases present a major problem throughout the world causing thousands of deaths each year from the consumption of food and water contaminated with toxin and pathogens. The most known organisms linked with these diseases include *Listeria monocytogenes*, *Salmonella* spp., Shiga toxin producing *Escherichia coli* (STEC), and *Campylobacter* spp. Food contamination can occur at any point of the food chain, from the raw material to the consumer's table. Pathogens are able to enter and contaminate the food system through humans, animals, air, water, soil and contaminated equipment. The increased level of knowledge on how, where, and when the contamination of food happens and how preventive measures should be applied present a necessity for the food safety of the products we consume in our everyday life.

Listeria species are widespread in the environment including in soil, raw foods, stream water, silage, sewage, plants, and animals. *L. monocytogenes* is a ubiquitous pathogen that can cause infections in humans, thus representing a major concern for the public health and economical aspect. The European Food Safety Authority reported 2536 confirmed human cases and 247 deaths in 2016, and 1887 confirmed cases with 166 deaths were reported in 2020. In 2021, the member states reported 2,183 confirmed invasive human cases of *Listeria monocytogenes* infection to ECDC. These cases resulted in 923 hospitalizations and 196 deaths in the EU where listeriosis was the fifth most reported zoonosis in humans and is one of the most serious foodborne diseases under EU surveillance (EFSA, 2017; EFSA, 2021). The annual number of listeriosis cases has increased significantly in the European Union since 2008.

Listeria species also commonly colonize the food processing environment and ready-to-eat products. Ready-to-eat foods are products consumed without any heat-treatment and often are associated with listeriosis outbreaks. *Listeria* species can colonize and persist in specific ecological niches within the food processing environment. A variety of hurdles such as heat treatment, disinfection, reduction of water activity, reduction of pH, or removal of oxygen, can be used to prevent or eliminate pathogens from foods. The response of foodborne pathogens to these stresses is a major concern because it can result in an enhanced survival and enhanced virulence of the pathogen.

It is estimated that up to 80% of bacteria on the Earth live in biofilms (Flemming and Wuertz, 2019). It has been suggested that biofilm formation is a stress response by bacterial cells, a result

of the preparation for unstable environmental conditions. In addition to providing protection against environmental stresses (e.g., heat, high salt concentrations), microbial biofilms also show increased resistance to disinfectants and antimicrobial treatments (e.g., antibiotics or preservatives).

In general, *Listeria* as a pathogen with different genetic survival mechanisms has the ability to withstand environmental factors such as heat, cold, salt and acidic conditions. The organism is ubiquitous in the environment and can grow in a wide variety of foods, including those stored at refrigeration temperatures. Elimination of this bacterium from ready-to-eat foods and food-processing equipment is difficult because they adhere and grow on food surfaces such as stainless steel (the most frequent material used in food-producing surfaces), rubber (extensively used in conveyor belts), glass (as a packaging material) and polystyrene (frequently used in the packaging of products). The main reason is because of the ability of this bacterium to form biofilms that protects it from stresses in food-processing environments, which can lead to reduced shelf-life of food as well as significant food safety hazards.

Environmental factors, including temperature, sugar, salt, pH, and nutrients that are common in foods and food-processing environments, have been demonstrated to have impacts on the adhesion and biofilm formation of *Listeria* strains. Among them, the most frequently studied abiotic environmental factors are temperature and osmolarity.

To understand the susceptibility of *Listeria* species biofilms to different stress conditions, fifteen biofilm forming *Listeria* strains were analyzed. Five of the chosen strains were non-pathogenic strains and ten of them were pathogenic (*L. monocytogenes* strains and *L. ivanovii*) which were taken in order to compare the difference of the susceptibility between the pathogenic and non-pathogenic strains to different stress conditions (applied separately and in combination) when grown under biofilm conditions.

Different treatments of chlorine, balsamic vinegar, acetic acid, white vinegar, and lactic acid were evaluated to determine the role of those solutions at the stage of washing lettuce to remove *L. monocytogenes*.

2. OBJECTIVES

The concept of this study derives from a *L. monocytogenes* outbreak of a Hungarian frozen vegetables company that sickened 54 people in six countries, resulting in fatality for 10 of them (EFSA and ECDC, 2018). The source of contamination was found to be the persistent presence of *L. monocytogenes* in one of the freezing tunnels of the factory. Such outbreaks demonstrate that there are still gaps in knowledge about the factors of the development of biofilms of food-borne pathogens such as *L. monocytogenes*.

The overall objective of my research was to better understand the influence of different environmental conditions on the biofilm formation of different *Listeria* species and to investigate the efficiency of some antimicrobial compounds against *L. monocytogenes* present in fresh produce (lettuce).

The specific objectives of my research are:

- to analyze the biofilm formation ability of different *Listeria* species,
- to investigate the differences on biofilm formation of different *L. monocytogenes* strains under different stress conditions,
- to compare the effect of stress conditions on biofilm formation of *L. monocytogenes* strains (NCAIM B1454, 3b T1, CCM5576, CCM7202, NCAIM B01966T, 11/4.12t03, 10887 ½ a, CCM 4699, and 5105 3a),
- to examine the efficacy of chlorine, acetic acid, balsamic vinegar, white vinegar, and lactic acid on the decline of biofilms of *L. monocytogenes* strains on the surface of lettuce.

3. LITERATURE REVIEW

3.1 *Listeria* species

3.1.1 Background of *Listeria* genus and listeriosis

Listeria genus includes Gram-positive, non-spore-forming, facultative-anaerobic rod-shaped bacteria. *Listeria* species can be separated in two groups based on the relatedness of species to *L. monocytogenes*. The first monophyletic group of *Listeria spp.* consist of six different species: *L. monocytogenes, L. ivanovii* including subspecies *ivanovii* and *londoniensis, L. innocua, L. welshimeri, L. seeligeri, and L. marthii,* which share similar phenotypic characteristics (e.g., motility, positive catalase reaction, ability to grow in low temperatures) and it is known as *Listeria* sensu strictu group. *Listeria* sensu lato is the other group which includes 15 *Listeria* species, known as non-pathogenic species, comprised of *L. grayi* including subspecies *grayi* and *murrayi, L. floridensis, L. fleischmannii* including subspecies *fleischmannii* and *coloradonensis, L. aquatica, L. rocourtiae, L. newyorkensis, L. cornellensis, L. grandensis, L. weihenstephanesis, L. booriae, L. costaricensis, L. goaensis, L. thailandensis, L. valentina and L. riparia* (Chiara et al. 2015 ; Carlin et al. 2021).

Listeria species usually are small rods ranging in size from 0.4-0.5 by 1-2 μm, but sometimes they can appear in short chains when are seen under the microscope. At room temperature, flagella can be detected, and motility can be observed in broth and semi-soft agar at 30 °C (Roberts et al. 2009). However, *Listeria* doesn't have flagella at temperature 37 °C, but when grown at 20 °C many flagella are distributed with random arrangement around the cell (Peel et al. 1988).

Listeria species are widespread in the environment, including soil, raw foods, stream water silage, sewage, plants, and animals. Two *Listeria* species, *L. monocytogenes* and *L. ivanovii* are considered pathogens. *L. monocytogenes* causes listeriosis in humans and animals, while *L. ivanovii* usually is associated with infections only in animals. However recently it was found out that *L. ivanovii* can cause infections also in humans (Guillet et al. 2010). *L. innocua* is a non-pathogenic strain that is used as a surrogate instead of *L. monocytogenes* in several studies because shares similar environments with *L. monocytogenes* (Rodríguez-Lázaro and Hernández, 2014).

L. monocytogenes is able to cause serious infections such as human listeriosis, a rare disease that is associated with high mortality rates. According to studies regarding products associated with outbreaks, the infectious dose seems to be 10^3 cfu/g levels.

Listeriosis commonly presents with symptoms such as fever, altered consciousness, rashes, respiratory distress, nuchal rigidity, headaches, anemia, elevated C-reactive protein (CRP), leukocytosis, and neutrophilia (Shi et al. 2021). Additionally, neurological symptoms like headache can also be a prominent feature, sometimes presenting as an isolated symptom in *Listeria* meningitis and encephalitis cases, emphasizing the importance of considering atypical presentations for accurate diagnosis (Mansell, 2022). The clinical manifestations of listeriosis can vary widely, making it crucial for healthcare providers to consider unexplained fever, altered mental status, and specific laboratory findings like elevated CRP and D-dimer levels to aid in early diagnosis and appropriate treatment interventions (Pérez-Pereda et al. 2020).

Listeriosis presents in various forms, including encephalitis, reproductive issues like abortion, mastitis, and ophthalmitis (Zakir et al. 2022). In humans, the most common forms are neurolisteriosis, bacteraemia, and maternal-neonatal infection, with symptoms ranging from febrile illness to severe neuropsychiatric manifestations (Pagliano et al. 2017). The disease can manifest as septicemia, neurolisteriosis, or focal infections associated with prosthetic devices (Prado et al. 2019). Additionally, in animals, listeriosis can manifest as encephalic, reproductive, or septicemic forms, with the nervous form primarily caused by *Listeria monocytogenes*. The clinical presentation of listeriosis can vary widely, from gastroenterocolitis to severe sepsis and multisystemic involvement, highlighting the diverse ways in which this bacterial infection can affect both animals and humans.

In humans, the most sensitive group, is considered to be the YOPI group that includes **y**oung (neonates), **o**ld (the elderly) people, **p**regnant women and people with impaired **i**mmune system such as transplant recipients, cancer patients, diabetics, and persons with AIDS (Adams and Moss, 2008).

Pregnant women as one of the most at-risk populations to get listeriosis, are 20 times more likely to be infected than other healthy adults. Listerosis in pregnant women can be asymptomatic or can be manifested by flu-like symptoms such as fever, myalgia, and headache (Frederiksen and Samuelsson, 1992). Listeriosis may lead to more serious consequences for the fetus, including spontaneous abortion, stillbirths, and sepsis in infants. According to studies, the fetus is more likely

than the mother to be affected with listeriosis thus having a high mortality rate around 80 percent (Charlier et al. 2017). While in pregnant women infections of the central nervous system are very rare, non-pregnant adults may develop a particular tropism in the central nervous system in cases of listeriosis (Rocourt et al. 2000).

Elderly individuals infected with *Listeria monocytogenes* commonly present with symptoms such as fever, headache, neck stiffness, altered mental status, and a longer prodromal phase compared to younger adults (Du et al. 2023). Additionally, symptoms may include respiratory failure, rapid progression, and easily confused clinical manifestations, leading to a low positive rate of cerebrospinal fluid culture (Pagliano et al. 2016). In some cases, elderly patients may experience focal infections like septic arthritis, vascular graft infection, or perianal abscess, which require a high index of suspicion for diagnosis (Chavada et al. 2014). Furthermore, central nervous system involvement by *Listeria monocytogenes* in the elderly can manifest as meningitis, meningoencephalitis, or even brain abscesses, with a propensity for misdiagnosis due to similarities with other conditions like neoplasms (Ogunleye et al. 2021). Early recognition of these symptoms and prompt treatment are crucial in reducing mortality and morbidity associated with listeriosis in the elderly (Wei et al. 2021).

According to annual projections, it was estimated that in the USA around 2,500 cases of listeriosis will occur, 500 of which will result on fatality and 300 of cases will require hospitalization (Mead et al. 1999). Furthermore, in 2014 in the United States, around 90 percent of the invasive *L. monocytogenes* infections require hospitalization and around 20-30% of listeriosis cases result in fatality. Moreover, from all the foodborne illnesses caused by pathogens such as *Campylobacter* or *Salmonella*, infections caused by *Listeria* are rare.

Campylobacter infection is a significant health concern globally, with varying incidence and severity across different age groups. Studies have shown that *Campylobacter spp*., particularly *C. jejuni*, are major causative agents, with a high incidence in both children and the elderly (Velev et al. 2022; Moffatt et al. 2021). Severe cases can lead to hospitalization, especially in individuals with underlying liver disease, older adults, and those with complications like bacteraemia. The infection typically presents with symptoms such as severe diarrhea, abdominal pain, and fever, with potential post-infectious functional gastrointestinal disorders in children (Pal, 2017). Antimicrobial resistance of *Campylobacters* is a growing concern, with high resistance rates observed in different age groups, emphasizing the need for prudent antibiotic use and control measures to mitigate the spread of resistant strains (Kiran et al. 2021). Understanding these factors

is crucial in managing and preventing the incidence and severity of *Campylobacter* infections. Although campylobacteriosis is more common than listeriosis, the mortality of the latter is much higher. Thirty-four deaths from campylobacteriosis were reported in 2022, resulting in an EU case fatality rate of 0.04%. The average percentage of fatal outcomes observed has remained unchanged over the past 5 years (range 0.03%–0.05%) compared to 10%-30% for listeriosis (Hernandez-Milian and Payeras-Cifre, 2014; EFSA and ECDC, 2022).

Salmonella infections pose a significant health burden in the United States, with varying incidences and severities documented across different age groups and infection types. Studies have shown that non-typhoid *Salmonella* infections can range from gastroenteritis to severe cases with bacteremia, with a decrease in seasonal variation noted as the severity of infection increases (Gharpure et al. 2021; Vugia et al. 2004). Incidence rates of invasive salmonellosis are highest among infants, with men having a higher incidence than women (Gradel et al. 2007). Additionally, antimicrobial resistance in nontyphoidal *Salmonella* infections has been associated with adverse clinical outcomes, with resistant culture-confirmed infections estimated to be around 6,200 annually (Gradel et al. 2007). Similarly to campylobacteriosis, the frequency of salmonellosis is much greater, but the mortality is much smaller compared to listeriosis (an average 10-30% compared to 0.03% (Hernandez-Milian and Payeras-Cifre, 2014; Cummings et al. 2010).

In the US the notification rate for listeriosis in 2010 was 0.27 cases per 100,000 population. This was like the rate of 0.28 cases per 100,000 population in 2009 (CDC, 2012). In the European Union (EU) there were 0.32 confirmed cases of listeriosis per 100,000 population in 2011. This was a 7.8% decrease in the number of cases from 2010. The reported fatality rate in the EU in 2011 was 12.7% (EFSA, 2013).

Listeriosis has shown a concerning upward trend in incidence over recent years, in Poland, from 2012 to 2021, listeriosis cases increased by 52.2%, with a median incidence of 0.23 per 100,000 population (Księżak and Sadkowska-Todys, 2024). The average annual incidence in Italy was estimated at 0.77 per 100,000, with significant underreporting noted (Ponzio et al. 2023) and Spain reported an average incidence of 1.55 per 100,000, highlighting the disease's impact on mortality, especially among the elderly (Pedro et al. 2022). The case fatality rate in Vojvodina, Serbia, was 23.08%, with the highest rates in individuals aged 19-59 (Jovanović et al. 2022).

3.1.2 *Listeria* in nature

All *Listeria* species are ubiquitous in nature and have been detected in various environments such as soil, sewage, vegetation, water, animal feed, fresh and frozen meat, slaughterhouse wastes and the faeces of healthy animals. According to studies aiming to isolate *Listeria* species from natural environments not associated with domestic livestock, higher frequencies of other *Listeria* genus members were detected comparing with *L. monocytogenes*. For example, a study reported a high incidence of *L. seeligeri* in samples taken from general environment (MacGowan et al. 1994). Whereas another study that included samples of grass, leaves, stems and roots, resulted in 9 of 10 positive *L. monocytogenes* isolates in wilting grass samples, but gave no presence of *L. monocytogenes* in the samples isolated from roots and stems (Fenlon et al. 1996).

Several studies have often failed to isolate *L. ivanovii* from environmental samples (Chapin et al. 2014; Fox et al. 2015). Sauders et al. (2012) reported that 23.4 and 22.3 % of samples obtained from natural and urban environments in USA, respectively, were positive for *Listeria*; the 442 *Listeria* isolates characterized in this study represented *L. seeligeri* (234 isolates), *L. monocytogenes* (80 isolates), *L. welshimeri* (74 isolates), *L. innocua* (50 isolates), and *L. marthii* (4 isolates).

In a study in Austria (Linke et al. 2014), 149 out of 467 soil samples (30 %) were positive for *Listeria* spp.; species identified included *L. monocytogenes* (6 % of all samples), *L. seeligeri* (15 %), *L innocua* (6 %), *L. ivanovii* (3 %), *L. welshimeri* (2 %), and unidentified *Listeria* spp. (2 %). *L. seeligeri* has been the most commonly isolated *Listeria* species in the two largest studies on *Listeria* diversity in natural environments (Sauders et al, 2012; Linke et al. 2014).

In a study of Stea et al. (2015), *Listeria* spp. were isolated from 53.8 % of the 329 water samples with a detection rate of 72.1 % in rural watersheds compared to 35.4 % for urban watersheds. *L. monocytogenes* was found in 30.3 and 34.5 % of the positive rural and urban watershed samples, respectively, and *L. ivanovii* was found in 8.4 and 6.9 % of these samples.

3.1.3 The occurrence of *Listeria* in food

3.1.3.1 *Listeria* species in food

Listeria species are a leading cause of bacterial-derived foodborne disease worldwide. Raw beef was found to be the predominant food item contaminated with *Listeria* species according to the study of Diriba et al (2021). Out of 369 food items of animal and plant origin examined, around (29.3%) raw beef meat was contaminated with *Listeria* species, in which *L. monocytogenes* rated 11.1%. *L. innocua* was the predominant *Listeria* species isolated from this food item by account 61.1% of the total *Listeria* species (Diriba et al. 2021).

According to the study of Vitas (2004). The high contamination of the beef samples may be due to poor hygienic conditions during slaughtering, processing and selling. This indicates that raw or undercooked beef meat may affects the public health by increasing the risk of diseases linked to *Listeria* species. Further processing and handling of meat also increases the risk of contamination with *Listeria* species.

A significant level of contamination of raw milk with *Listeria species* (23.3%) was identified in Ethiopia. *L. monocytogenes* was the predominant bacterium isolated from raw milk with prevalence of 25.6% followed by *L. welshimeri*, *L. seeligeri* and *L. ivanovii* with prevalence ranged from 14 to 21% (Diriba et al. 2021). This high prevalence of *Listeria* species in milk might be due the tradition of mixing milk with water before selling to the consumer which increases the chances of contamination during dilution with water, poor personal hygiene or from contaminated environment and poor milking practices (Peeler et al. 1994).

Listeria sensu lato is comprised of *L. grayi* as well as *L. fleischmannii, L. floridensis, L. aquatica, L. newyorkensis, L. cornellensis, L. rocourtiae, L. weihenstephanensis, L. grandensis, L. riparia, and L. booriae.* Phylogenetically, *L. grayi* is most closely related to *Listeria* sensu strictu species. *L. fleischmannii, L. floridensis,* and *L. aquatica* share a most recent common ancestor with *L. grayi* and sensu strictu species, while the other *Listeria* sensu lato species group together in the most basal cluster within the genus (Weller et al. 2015). A list of *Listeria* sensu lato species with their source of isolation is provided in Table 1.

Table 1. Isolation locations of *Listeria* sensu lato species (Orsi and Wiedmann, 2016)

Species	**Source of isolation**
L. rocourtiae	Pre-cut lettuce, Salzburg (Austria)
L. weihenstephanensis	Vegetation (Lemma trisulca) from pond in Wolnzach/Pfaffenhofen (Germany)
L. fleischmannii	Cheese and ripening cellars (Switzerland); cheese (southern Italy); environmental samples, cattle ranch, Colorado (USA)
L. floridensis	Running water, Florida (USA)
L. aquatica	Running water, Florida (USA)
L. cornellensis	Water, Colorado (USA)
L. riparia	Running water, Florida (USA)
L. grandensis	Water, Colorado (USA)
L. newyorkensis	Non-food-contact surface in a seafood processing plant (northeastern USA); raw milk (southern Italy)
L. booriae	Non-food-contact surface in a dairy processing plant (northeastern USA)
L. grayi	Various locations (worldwide)

3.1.3.2 *Listeria monocytogenes* in food

L. monocytogenes has been isolated from a wide range of foods and all of them have been associated with major outbreaks. Contaminated foods with *Listeria* include both raw and processed foods. *L. monocytogenes* can be isolated from many foods such as beef, pork, fermented sausages, fresh produce, and fish products but also from other foods such as soft cheeses, hot dogs, and seafood who have been repeatedly involved in listeriosis outbreaks (Rocourt and Cossart, 1997). According to data collected from studies on sporadic and epidemic listeriosis cases, it appears that there are certain foods that posses higher risk than others. Mainly, the highest risk of contamination is associated with ready-to-eat foods, which are industrially processed foods and require storage at low temperatures for a long time, thus enabling *Listeria* to grow and reach the infective doses (Rocourt et al. 2000). In a study from Huzhou, China, a 3.7% prevalence was noted in retail foods, with seasoned raw meat showing the highest contamination rate at 15.2% (Zhang et al. 2024), and

L. monocytogenes was detected in 6% of food samples from food-processing environments, with high contamination rates in products like meats and cheeses (Jordan and McAuliffe, 2018).

According to the European Commission Regulation No. 2073/2005, there are different established microbiological criteria for the prevalence of *L. monocytogenes* for ready-to-eat foods depending on the intended consumption. The three proposed categories are as follows: "(i) absence of bacteria in 25 g product for infants and patients with special nutritional needs and <100 cfu/g product for people outside of these groups, (ii) absence in 25 g before the product leaves the food operator and <100 cfu/g during the shelf life of the product for the products which support the growth of *L. monocytogenes*, (iii) lower than 100 cfu/g in products who don't support the growth of *L. monocytogenes"*.

Table 2. shows the growth and survival limits of *L. monocytogenes* (Food Safety Authority of Ireland 2005). According to the table below, *L. monocytogenes* shows a wide range of optimal conditions for growth and survival during food processing and storage comparing with other bacteria.

Table 2. Growth and survival limits of *L. monocytogenes* (Food Safety Authority of Ireland 2005)

Parameter	Range	Optimal[d]	Can survive (but no growth) [e]
Temperature (°C)	-1.5 to 45	30 to 37	-18
pH[a]	4.2 to 9.5	7	3.3 to 4.2
Water activity(aw)[b]	0.90 to >0.99	0.97	<0.90
Salt (%) [c]	<0.5 to 12	N/A	≥20

a. Hydrochloric acid as acidulant (inhibition is dependent on type of acid present).
b. Sodium chloride as the humectant.
c. Percent sodium chloride, water phase.
d. When growth rate is highest.
e. Survival period will vary depending on the nature of the food and other factors.
N/A Not applicable.

It can be seen from Table 2, that a neutral environment with a temperature range from 30 to 37 °C is considered to be the most optimal condition for *Listeria monocytogenes*. However, *L. monocytogenes* can survive even at refrigeration temperatures (less than 5 °C) during food storage. But despite the wide range of growth conditions, many studies can be found in the literature on how to control *L. monocytogenes* in the food-processing industry and understand its behaviour in the food systems. According to Tompkin (2002), *Listeria* control program include the following strategies regarding ready to eat (RTE) foods: "(i) prevention of the establishment and growth of *listeriae* in niches or other sites that can lead to the contamination of RTE foods; (ii) implementation of a sampling program that can assess in a timely manner whether the environment in which RTE foods are exposed is under control; (iii) as rapid and effective a response as possible to each positive product contact sample; (iv) verification by follow-up sampling that the source has been detected and corrected; (v) a short-term assessment (e.g., involving the last four to eight samplings) to facilitate the detection of problems and trends; and (vi) a longer-term assessment (e.g., quarterly, annually) to detect widely scattered positive sites on a packaging line and to measure overall progress toward continuous improvement".

3.1.4 *Listeria* in food processing plant

3.1.4.1 *Listeria species* in food processing plant

Listeria species isolated from food processing plants include *L. seeligeri, L. innocua, L. ivanovii,* and *L. welshimeri*, was identified in various studies. These species have been detected in different regions such as Bavaria, Germany, South Africa, China, and Poland, reveal a global presence in food processing environments (Voronina et al. 2023; Wartha et al. 2023; Mafuna et al. 2022; Liu et al. 2022; Wiśniewski et al. 2022).

The main reason that *Listeria* species present serious concern for many manufacturers on contamination of industrially processed foods appear to be the persistence of *Listeria* species in the food processing environment but also the food processing conditions and the environment that support *Listeria* to grow. This persistence and good growth properties are the consequences of its characteristics. *Listeria* species are Gram-positive, facultative anaerobe, intracellular rod bacteria which growth is supported by wide pH range conditions (4.3-9.5), high salt tolerance (up to 20%), growth at low temperature, relatively low water activity (> 0.90), resistance to different stress

conditions and biofilm forming ability. Such conditions enable *Listeria* to survive and grow in different food and food processing environments (Farber and Peterkin, 1991).

Many studies have demonstrated that specific strains of *Listeria* spp. have the ability to colonize, multiply, and persist in the food processing environments as well on the food processing equipment over extended period and become potential contaminants for food during processing or after processing. According to the study of El-Shenawy (1998), the presence of *Listeria spp*. in food processing environment including food handlers was investigated in 19 food processing units (the unit represents a part of big dairy plant) in addition to two other plants that produce frozen and non-frozen foods. *Listeria spp.* was detected in all investigated food processing units and were isolated from direct sources such as drains, floors, standing/condensed water, process equipment, miscellaneous and residues.

3.1.4.2 *Listeria monocytogenes* in food processing plant

The growth conditions of *Listeria monocytogenes* mentioned in chapter 3.1.3.2 made this bacterium able to survive and multiply in adverse environmental conditions which are often present at food production facilities (Gray et al. 2006). It has been demonstrated that *L. monocytogenes* can survive in the environment for at least 8 weeks (Rodríguez-Campos et al. 2019). Several investigations have shown that *L. monocytogenes* is widely distributed in food processing environments where it is able to persist for a long time due to ineffective cleaning and sanitation (Carpentier and Cerf, 2011).

L. monocytogenes has the ability to attach to many food-contact surfaces in food industries such as stainless steel, polystyrene, and glass that may directly or indirectly come in contact with food and cause contamination. Many strains survive in different food processing conditions which are often characterized with a low humidity or oxygen content and thus becoming a main source of post-processing contamination (Hoelzer et al. 2012; Ferreira et al. 2014).

Mainly the industrially processed foods that require storage at low temperature known as ready-to-eat (RTE) foods are linked to these infections. There are several cases that have been reported, where it was found that *L. monocytogenes* contaminated industrially processed foods such as cheese and meat (Martin et al. 2014; Rückerl et al. 2014). Persistence of *L. monocytogenes* may

be contributed by several external factors as poor hygiene practice or ineffective sanitizers (Nilsson et al. 2011; Rodríguez-Campos et al. 2019).

3.2 Biofilms

Microorganisms in environment appear in two types of forms, either as planktonic cells or as communities in biofilms. Biofilms are attached to the surface and enclosed to a self-produced matrix of extracellular polymeric substances (EPSs) that is mostly composed of proteins, polysaccharides and DNA. EPSs have several important features to biofilm such as higher resistance to removal and destruction and an increased resistance to antimicrobials.

Microbial cells are responsible for adhesion to surfaces and cohesion in biofilm (Flemming et al. 2007). Biofilm formation allow bacteria to survive in unfavourable conditions and be persistent in food processing environments. In case when biofilms contain pathogenic bacteria, it could cause contamination of food products and food-borne outbreaks.

Based on the research paper of Vestby and co-workers 2020, biofilm may potentially contribute to the pathogenesis of a disease in several ways. Biofilm formation increases the bacteria's resistance against the defense mechanisms of the body, as well as antimicrobial treatments, thereby promoting chronic infections. Biofilms may also function as an environment that accumulates different bacterial species as well as bacterial numbers in certain locations. This can result in deleterious effects on host cells due to concentrated, sequential, and/or synergistic activities by the present bacteria. Furthermore, the mere presence of persistent biofilms may modulate the local immune response in several ways, e.g., by stimulating a local inflammatory response that can cause or aggravate tissue damage. These biofilm-mediated mechanisms have also been suggested to be involved in the initiation and/or progression of cancers.

3.2.1 Development of biofilms

The process of formation of a bacterial biofilm can be considered as a process that contain five developmental stages, as it can be seen in Figure 1.

The five stages of the biofilm development are the following:

1. Reversible attachment of planktonic cells to surfaces.
2. Production of extracellular polymeric substances (EPSs) that results in more firmly adhered “irreversible” attachment.
3. Development of biofilm and replication of the cells within the self-produced extracellular polymeric (EPSs) matrix.
4. Biofilm maturation, when cells irreversibly are attached to the surface and enclosed in the matrix.
5. During detachment cells are dispersed from the biofilm and return to planktonic form ready to colonize another surface to re-initiate the biofilm formation again (Van Houdt and Michielis, 2005).

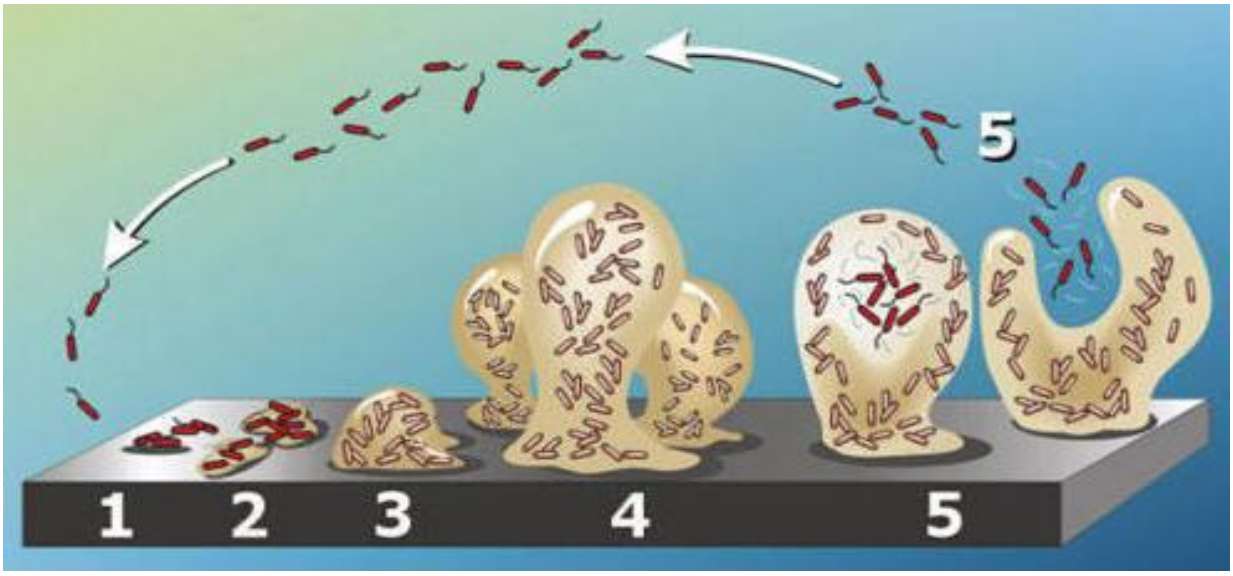


Figure 1. Process of biofilm formation: 1- Attachments of cells, 2- Cells proliferate and extrapolymeric substances (EPSs) are formed, 3- A three-dimensional structure forms, 4- Maturation and 5-Deattachment of cells with recolonization (Van Houdt and Michielis, 2005).

3.2.2 *Listeria* biofilm formation ability

3.2.2.1 *Listeria* species biofilm formation ability

Listeria species have the ability to form biofilms on different materials used in the food industry. However, according to studies, hydrophilic surfaces such as stainless steel and glass have been

shown to be much more efficient on attachment of bacteria than hydrophobic surfaces such as polystyrene. The main factors that affect the development of biofilm formation are the surface, strain, temperature and medium (Di Bonaventura et al. 2008). One of the materials, most commonly used in the food industry, is stainless steel that allow biofilm formation of many bacteria including *Listeria* strains. It is estimated that cross-contamination related to surfaces concerns 39% of outbreaks of food-borne diseases (Evans et al. 1998).

The adhesion of *Listeria* spp. strains to biotic surfaces and the emergence of biofilm is widely documented, e.g. in meat and dairy processing (Simões et al. 2010). Bacteria in the biofilm structure are more resistant to disinfectants and UV radiation which make it difficult to eliminate from food plants (Davidson and Harrison, 2002).

L. innocua was found in drain, in grating and on the floor in front of slicer, *L. welshimeri* was isolated from floor, *seeligeri* were isolated from the slicer, control panel (Shimojima et al. 2023). *Listeria grayi* was isolated from supermarket food-contact surfaces (Ríos-Castillo et al. 2021).

3.2.2.2 *Listeria monocytogenes* biofilm formation ability

The formation of biofilms is considered to be a major problem for the food industry because it can be a source of contamination of food materials or foodstuffs that come into contact with them, which lead to food spoilage or foodborne diseases. For example, potential sources of contamination by *L. monocytogenes* are environmental surfaces such as walls and floors, where people, air and cleaning agents have the role of transmission vectors of microorganisms to the food. Such contamination is derived by the accumulated biofilms on surfaces such as floors, wastewater pipes, conveyor belts, rubber seals, and stainless steel, as well as from improper cleaning and sanitizing of the equipment and airborne microbiota (Di Ciccio et al. 2012).

According to studies, *L. monocytogenes* is less sensitive to biocides within biofilms comparing with planktonic form. Since biofilms generally protect the cells from antimicrobial action, adhered bacteria are more resistant to cleaning and disinfection than the bacteria in a suspension (planktonic cells) (Simões et al. 2009; Aarnisalo et al. 2000; Chavant et al. 2004). It was, therefore, suggested that the resistance of *Listeria* to antimicrobials in food processing environments is linked to the ability of the cells to attach and to form biofilms (Pan et al. 2006; Van der Veen and Abee, 2011), however strains differ in their adherence or biofilm forming ability (Harvey et al.,

2007). The resistance of *Listeria* to the action of biocides is influenced by several factors, including physical and chemical properties of the surface, temperature, growth medium, growth phase of the bacterium, cell characteristics, the presence of food residues and other microorganisms (Chavant et al. 2004, Pan et al. 2006). There is, therefore, a great need to adopt best cleaning and disinfecting practices against *Listeria*.

In general, antimicrobials have been shown to be very effective in reducing and inactivating microorganisms. However, there are still risks because of the chance of regrowth of the cells and by the ability of bacteria to form biofilms that could lead to the increase of resistance of *Listeria* to antimicrobials (Minei et al. 2008). These facts show that biofilms still represent a major problem in the food industry.

Bacteria are able to colonize and form biofilms on almost all kinds of surfaces, including natural and synthetic surfaces. Biofilms are responsible for chronic illnesses and nosocomial infections, industrial pipe fouling, spoilage of foods, contamination of sea food and dairy products, as well as ship hull fouling. Some of the biofilm-forming pathogenic and potentially pathogenic microbes include *Aeromonas hydrophila, Burkholderia cepacia, Enterococcus faecalis, Escherichia coli, Haemophilus influenza, Klebsiella pneumoniae, Listeria monocytogenes, Pseudomonas aeruginosa, Pseudomonas pseudomallei, Proteus mirabilis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus mutans, Streptococcus viridans, other Streptococcus species, Vibrio cholerae, Vibrio parahaemolyticus,* and *Candida albicans* (Abee et al. 2011; Bowler et al. 2020; Han et al. 2016; Jahid et al. 2015; Khatoon et al. 2018).

Further biofilm forming microbes include *Bacilli* (*Bacillus subtilis, Bacillus cereus*), *Lactobacillus plantarum, Lactococcus lactis,* and *Lactobacillus rhamnosus* (Abee et al. 2011). There are a few pieces of evidence provided on biofilm-forming moulds species, and in recent years, some genera of pathogenic fungi have been gaining attention and are correlated with biofilm formation (Bowler et al. 2020). In most conditions, bacteria will generally grow on surfaces in competition with other microorganisms in a mixed species of biofilm (Abee et al. 2011). Therefore, the harmful effects of biofilms on human society are manifold. The most common biofilm-forming foodborne pathogens and spoilage organisms are introduced in Table 3.

Table 3. Representatives of foodborne bacteria that form biofilms (Muhammad et al. 2020)

Foodborne Bacteria	Growing Substrate	Spoiled Food	Genes Related to Biofilm Formation
Bacillus (Bacillus cereus)	Stainless steel, plastic, soil, and glass wool	Sprouted seeds, fruit juices, fried rice, pasta dishes, meat products, vegetables, and milk products	*tasA, galE, eps2, mogR, comER, plcR, rpoN, codY, spo0A, abrB, sinI, sinR* and others
Clostridium	Multi-species biofilm	Dairy products, fish, cattle meat, poultry, vegetables, honey, and canned food	*luxS, spo0A, pilC, pilT,* and others
Cronobacter spp.	Powder service and powder packaging rooms, spray-drying areas, and evaporator rooms	Dairy products, vegetables, grains, bread, herbs, sausages, spices, and meat	*bcsR, csgA, csgB* and others
Escherichia coli	Stainless steel surfaces, food contact surfaces	Dairy products, fermented meat sausage, meat, poultry, fish products, drinks, and vegetables	*fim, pap, bfp, scg, sfa, foc, afa, flu, pgaABCD, bcsABZC, uvrY, csrA* and others
Listeria monocytogenes	Wastewater pipes, floors, conveyor belts, rubber seals, elastomers, and stainless steel	Dairy products, melons, coleslaw, ready-to-eat meat products, and ready-to-eat fish products	*luxS, agr (agrABCD), inlA, actA, prfA* and others
Pseudomonas spp. (Pseudomonas aeruginosa)	Conveyor belts, floors, drains, slicing and milking machines	Dairy products, red meat, and poultry	*psl (pslA–pslO), pel (pelA–pelG), algD, algU, algL, ppyR, lasR, lecA, rhlI, pilA, pilT* and others
Salmonella	Stainless steel, elastomers, concrete, glass, and food surfaces (such as lettuce and tomato)	Poultry, pig, cow meats, and dairy products	*bapA, csgB, csgD, csgBA, adrA, bcs, fimA, fimH, luxS, flgE* and others
Staphylococcus (Staphylococcus aureus)	Stainless steel, plastics (such as polystyrene and polypropylene), and glass	Dairy products, ready-to-eat meat products, ready-to-eat fish and seafood products, and ready-to-eat dairy products	*icaA, icaD, icaB, ica, icaR, fib, cna, fnbAB, clfA, clfB, agr (agrA-agrD)* and others

3.2.3 Methods of biofilm control

The risk of food contamination is increased by the prevalence of biofilm; thus, prevention of biofilms formation is an important step in the food industry. In order to reduce or remove the biofilms found on contact surfaces, physical and chemical methods of cleaning and disinfection have been used for many years. The process of removal of biofilms has been shown to be difficult due to the increased resistance to disinfectants, caused by factors such as the age of the biofilm and different stress responses (Van Houdt and Michiels, 2010).

According to studies, control of the operating time between cleaning and sanitation has been shown to be an important aspect to prevent biofilm formation in the pasteurization lines of dairy plants. Lindsay and co-workers (2005) recommended short intervals of cleaning and sanitation of food-processing surfaces to prevent or reduce biofilm formation of *Bacillus subtilis*. An adaptive response of microorganisms towards disinfectants results from the application of sub-lethal concentrations of disinfectants and after poor rinsing in the food processing environments, that could lead to an increased tolerance for the disinfectants.

The low-pressure cleaning system proved to be not so efficient in elimination of all the bacterial cells from the surfaces and did not kill the bacteria even after use of the strong alkaline cleaner. Removal of biofilms in food industry is difficult, but the application of good routines of biofilm control guarantees an efficient process that lead not only on killing the biofilm bacteria but also on the removal of polymer matrix of surfaces that prevent the recolonization of the cells (Bredholt et al. 1999).

3.2.3.1 Application of enzymes

Promising approaches can be based on the application of “green” and biological-based products, such as enzymes. Enzymes are applied in many fields of investigation and are known to be capable of degrading key components of the biofilm matrix. Moreover, it is recognized that the combination of enzymes with other biofilm control agents can enhance their activity. However, enzymes are specific, and to degrade the complex matrix that involves the biofilm, a mixture of enzymes is often necessary (Borges et al. 2020).

Some enzymes such as protease, DNase I, alginate lyase, amylase, and cellulase have been reported to support biofilm removal (Stiefel et al. 2016). Therefore, inclusion of these enzymes in cleaning agents can improve the efficiency of biofilm detachment. A few enzymatic cleaners are commercially available, but they often failed to show the expected biofilm removal efficacy in practice (Sava 2005). One of the reasons for failure is the use of inappropriate test parameters during the cleaner development process, which might lead to an overestimation of the cleaning performance, e.g., relevance of the used microorganisms, biofilm formation conditions, or readout of biofilm removal.

3.2.3.2 Phase transfer catalyst_ Sterilix

Examination of the literature revealed a proprietary product that is used in the food industry as an effective surface cleaner of biologic debris (Kramer and Shaw, 1994). The cleaner is based on the powerful nucleophilic hydroperoxide ion (HPI) in conjunction with phase transfer catalysts (PTC) and activators (Sterilex). The enhanced nucleophilic activity is ascribed to an alpha effect that imparts a high density of negative charge on the terminal oxygen of the peroxide ion. The net effect is the hydrolysis of biologic molecules containing ester linkages such as lipids and the amide linkages of proteins (Shepherd et al. 2001).

A major reason most proposed cleaners and disinfectants do not effectively remove biofilms is that both the cleaner or disinfectant and the biofilm carry a net negative charge, which results in repulsion or non-interaction of the materials. The phase transfer catalyst in HPI-PTC eliminates this problem and is able to penetrate the biofilm: Once inside the biofilm, the hydroperoxide ion is released, where it hydrolyzes and lifts the contaminating material away from the surface. The cleaner also has disinfecting properties (Shojaei and Staat, 1998).

3.2.3.3 Surface modification of contact material

The complexity of particular parts of processing equipment (joints) or bacterial cells “hidden” in cracks and scratches appearing on the surface do not guarantee complete sanitization (Sinde et al. 2000). Therefore, it is a valuable strategy to have the initial bacterial attachment on the surface of materials under control.

In food processing plants (e.g., brewery, water pipes), copper surfaces are still in use. It has undeniable anti-biofilm properties due to the interaction of copper ions with the cell membrane, causing the formation of reactive oxygenic species, DNA damage, and the impairment of DNA integrity (Gomes et al. 2020). However, the application of copper material is limited due to the high cost, possible leakage of copper ions, and corrosion.

Since stainless steel is a widely used material in the food industry, the environmental factors in food processing environments and the properties of stainless steel enabling the attachment of bacterial cells on the surface of SS have been intensively reviewed (Dula et al. 2021). Silver ions are known to interact with thiol groups of cysteine, resulting in the disruption of the permeability of membranes, and the release of a reactive oxygen species causing oxidative stress. Ag-doped stainless-steel surfaces were prepared using various technological processes including sol-gel, immersion in nanoparticles suspension, ion beam technology, spray coating, or simple Ag+ adsorption from water disinfectant (Li et al. 2019). However, coating procedures are often too complicated, comprising a number of organic substances, solvents, and accompanying chemical reactions.

3.2.3.4 Natural compounds as biofilm inhibitors

Various antimicrobial agents were assessed to prevent the adhesion of bacterial cells onto stainless steel surfaces via interrupting their metabolic pathways that lead to membrane damage, protein and cell wall binding, enzyme inactivation (Mishra et al. 2020). It is important to say that antimicrobial and anti-biofilm formation properties must be distinguished. It was previously described that tea polyphenols did not affect planktonic growth of *Shewanella putrefaciens* but were more effective in the inhibition of their initial attachment and the metabolic activity of the biofilm (Zhang et al. 2016).

Plant-based extracts and essential oils (Nazareth et al. 2021), as well as individual phenolic constituents (Walczak et al. 2021), are very attractive as antibiofilm-forming agents. The mode of action against bacterial cells can be antimicrobial (disrupting cell membrane), anti-QS activity (downregulate the transcription of genes involved in various metabolic pathways), altering the hydrophobicity of cells' surface, or their combined effect.

Oregano essential oil (OEO) significantly reduces biofilm formation of *L. monocytogenes* achieving a 62.03% decrease in biofilm coverage on glass slides compared to untreated controls (Guo et al. 2024). This study demonstrated that OEO not only inhibits planktonic bacterial growth but also effectively disrupts biofilm integrity. This suggests that OEO has potential as a natural antimicrobial agent for controlling *L. monocytogenes* biofilms, which are a concern in food safety and sanitation.

Glabridin, extracted from liquorice roots, a natural antimicrobial, effectively reduced biofilm cells of *L. monocytogenes*. Applying 200 µg/mL of glabridin resulted in a significant reduction of biofilm cells, achieving over a 3 $\log_{10}$ reduction under standard conditions. However, the presence of residual organic matter from food sources, particularly from smoked salmon and skim milk, substantially affected glabridin's disinfectant activity, indicating that the type of food matrix can influence its effectiveness against biofilms (Bombelli et al. 2024).

Natural aromatic compounds (e.g., thymol, eugenol), in combination with a *Listeria*-specific phage cocktail, significantly reduced the biofilm cell population of *L. monocytogenes*. The treatment decreased biofilm on contaminated celery by more than 2 log CFU/g and on food contact materials by over 2 log CFU/cm². Additionally, the combination treatment inhibited biofilm formation and downregulated virulence-related genes, demonstrating its potential as an effective antimicrobial and antibiofilm agent in the food industry (Byun et al. 2024).

Vinegar, particularly in the form of acetic acid, has shown potential as an antimicrobial agent against *L. monocytogenes*. Acetic acid concentrations of 1% and 2% significantly reduced *L. monocytogenes* count on poultry skin, demonstrating a decrease of approximately 1.31 log units after eight days (González-Fandos and Herrera, 2014). Additionally, the exposure to subMIC concentrations of white vinegar significantly decreased the biofilm-forming abilities of *L. monocytogenes* isolates after seven days, particularly at 4 °C and 37 °C, indicating that vinegar can negatively impact biofilm formation in these bacteria (Yüksel and Şen, 2023).

3.2.3.5 Microorganisms for pathogen biofilm control

It is known that various fungi, yeasts, or bacteria successfully inhibit the formation of biofilm. Such microorganisms are producers of natural compounds which act as antimicrobial agents against both planktonic cells and can also penetrate into the biofilm matrix. Nisin, a bacteriocin

produced by some strains of *Lactococcus lactis*, is widely used as an antimicrobial agent in the food industry. The anti-biofilm properties of nisin have been successfully examined against *Listeria monocytogenes* on stainless steel surfaces, where significant inhibition (4.6 log CFU/cm^2) has been observed (Minei et al. 2008).

After growing various microorganisms in culture media, cell-free supernatant was obtained and used against biofilm-forming bacterial species. For instance, crude extracts of *Actinomycetes* isolates inhibited the biofilm formed by *Bacillus cereus* and *Shewanella putrefaciens* on a stainless-steel surface (Mulya and Waturangi 2021). *Bacillus* sp. cell-free supernatant exhibited anti-biofilm activity against important fish pathogens (Santos et al. 2021).

3.2.3.6 Cleaning

The process of regular cleaning is widely used for removal of product residues and represent the first critical point on removal of biofilms. For an efficient process of cleaning is important that the cleaning agent should be chosen properly. Disinfectants are less effective when the surfaces are not clean, so it's very important to remove the food components that may lead to bacterial attachment and biofilm formation (Sinde and Carballo 2000).

The key parameters that effect the outcome of the cleaning process are appropriate formulation, time, temperature, concentrations of the applied agent, and mechanical forces applied. However, the change of one parameter can effect the other parameter, e.g. increasing of the concentration of the cleaning agent would decrease the application time (Van Houdt and Michiels 2010).

3.2.3.7 Sanitary design

Sanitary design is the application of design techniques that allow the effective cleaning of the entire manufacturing environments, so that the growth of bacteria is inhibited. Moreover, rational equipment design facilitates cleaning and cleaning-in-place processes that reduce the bacterial contamination of the food-processing equipment. Utilization of sanitary equipment design that do not allow areas that may be difficult to clean is one of the best ways to prevent biofilm formation. *L. monocytogenes* has been found in food processing environments even after cleaning (Lundén et al. 2003; Soumet et al. 2005). Persistance of *L. monocytogenes* is possible in sites of food-processing environments where cleaning and disinfection processes cannot be applied such as drains equipment and cracks in surfaces (Heir and Langsrud, 2014).

3.2.3.8 Chemical control

One of the most used strategies in the food industry to control biofilms is the chemical control. There are different types of chemical disinfectants which are used to prevent growth of microorganisms on surfaces of food processing environments, such as oxidizing agents, surface-active compounds and iodophores. The most used disinfectants include chlorine-based compounds, ozone, hydrogen peroxide, peracetic acid, quaternary ammonium compounds and acid ionic compounds.

The efficiency of disinfectants is usually influenced by factors such as contact time, temperature, pH, concentration, water hardness and interfering organic particles. Compounds of food particles, such as carbohydrates, proteins and fats can affect the efficiency of the disinfectants. An example of such influence is the inactivation of hypochlorite by proteins. In order to increase the disinfection efficiency in the food industry usually the cleaning agents like detergents are combined with disinfectants.

Food contact surfaces are cleaned and sanitized daily in most food processing plants, however many environmental surfaces such as storage tank and pump exteriors and walls are cleaned infrequently. This infrequent cleaning can lead to biofilm formation if moisture is present. However, an effective cleaning procedure should take place to break up or dissolve the EPS matrix associated with the biofilm so that sanitizing agents would have access to the viable cells. The impairment of heat transfer and corrosion to metal surfaces can come as a result of biofilm formation. Also, the application of hot water proves to be one of the most effective methods for biofilm control (Chmielewski et al. 2003).

3.2.4 Environmental conditions of biofilm formations

Biofilm formation by *L. monocytogenes* is influenced by a multitude of environmental factors, such as temperature, pH, aw, salinity, nutrient content, cell surface properties and cell structure (Cole et al. 1990). There are many studies focusing on biofilm-forming ability of food-borne pathogens to different environmental factors, cell characteristics and substrate properties (Renner and Weibel, 2011).

3.2.4.1 Temperature

Temperature plays an important environmental factor that influences the biofilm formation and favours the nature of adhesion to the surface and its hydrophobicity (Midelet et al. 2006). Therefore, many studies were conducted at high and low temperatures of food processing environments, in which conditions biofilm bacteria resulted to be very resistant thus becoming very difficult to eradicate (Cole et al. 1990). For this reason, biofilm bacteria still posses a major challenge to the food processing industry.

Research indicates that optimal temperatures can enhance biofilm development, while extreme temperatures may inhibit it. For *Aspergillus flavus*, optimal biofilm formation occurs at 37 °C, with increased conidial germination and resistance to antifungal agents at higher temperatures (42 °C) (Hernández-Benítez et al. 2025). For *Campylobacter jejuni*, temperature significantly influences biofilm formation with higher counts and stronger biofilm density observed at 42 °C compared to room temperature (Pokhrel et al. 2024), and according to Lin et al. (2024), low temperatures significantly inhibit biofilm development, reducing it by approximately 66% at 4 °C and 55% at 15 °C compared to 28 °C due to decreased microbial activity and communication.

Most studies of biofilm formation were conducted at 37 ± 2 °C which is the optimum growth temperature of *Listeria monocytogenes*, and at 25 °C which represents the temperature at which this microorganism forms flagella. According to a study that analyzed biofilm formation of 44 strains of *L. monocytogenes* on different surfaces (glass, polystyrene, and stainless steel) at four temperatures, at 4, 12, 22 and 37 °C, respectively, different organization of *L. monocytogenes* biofilms were observed. At 22 and 37 °C a complex organization of biofilms with high cell number and EPSs production were detected, whereas at 4 and 12 °C only low levels of biofilms consisting of sparse clusters and few EPSs (Di Bonaventura et al. 2008). According to researchers these results are not due to a cellular physiology difference but due to a reduced growth of bacteria. This study has shown that *L. monocytogenes* forms higher levels of biofilms at 4 and 12 °C on glass compared with polystyrene and stainless steel. However, at 37 °C, *L. monocytogenes* biofilm formation was significantly higher on glass and stainless steel compared with polystyrene.

Some studies have reported that *L. monocytogenes* can grow at low temperatures such as -1.5 °C but also at high temperatures such as 50 °C (Cole et al. 1990; Ryser and Marth. 2007). Low

temperatures that are used during food processing and storage enhance the risk of cross-contamination due to *L. monocytogenes* ability to form biofilms at such temperatures.

The effect of sublethal temperatures on the biofilm of *L. monocytogenes* is significant, influencing both its formation and resistance to environmental stresses. Research indicates that exposure to sublethal temperatures can enhance the biofilm's resilience, impacting its overall virulence and adaptability. According to Ágoston et al. (2010), pre-exposure to sublethal temperatures (e.g., 46 °C) significantly increases the heat resistance of *L. monocytogenes* suggesting that biofilms can withstand higher temperatures after such exposure. This adaptation may be due to metabolic changes and the protective role of extracellular polymeric substances in biofilms (Ekonomou and Boziaris, 2024).

The ability of *L. monocytogenes* to form resilient biofilms at sublethal temperatures poses challenges for food safety, as these biofilms can survive sanitation processes (Yüksel and Şen, 2023). Understanding these adaptations is crucial for developing effective control measures in food processing environments.

The interaction between temperature and other environmental factors, such as NaCl concentration and pH, also plays a role in biofilm formation, with higher temperatures generally promoting adhesion and biofilm development (Hu et al. 2022). Depending on the previous understandings, more studies on the influence of environmental conditions such as temperature on biofilm forming ability of *L. monocytogenes* would provide more information on advancing the food safety, such as information on inhibition conditions for biofilms that could be used as a tool to control the biofilms in food industry.

3.2.4.2 Salting

Salt concentration in particular sodium chloride concentration plays an important role on biofilm forming ability of *L. monocytogenes*. The effect of sodium chloride on biofilm formation was observed in case of different food-borne pathogens including *L. monocytogenes* and it was suggested that the number of adherent pathogenic cells has decreased with increasing of NaCl concentrations from 0% to 10% (Xu et al. 2010). A decrease of maximum growth rate and specific growth rate of adherent pathogenic cells with increasing sodium chloride concentration was

observed. Significant reduction in the numbers of adherent cells was observed also at low concentration levels of NaCl (<2%).

Moderate concentrations of salt (2-5%) can enhance the biofilm formation of *Listeria monocytogenes*. In these concentrations, salt acts as a stressor that triggers the activation of genes responsible for producing extracellular polymeric substances (EPS), which are essential for biofilm formation (Tresse, 2014). The study of Di Bonaventura et al. (2008) showed that the biofilm formation is enhanced also by cell surface hydrophobicity and auto-aggregation thus giving a better understanding on the adhesion and detachment capability of *L. monocytogenes* on food contact surfaces. This adherence is a key step in biofilm development, as it allows the bacteria to colonize surfaces more effectively.

According to Jiang (2017), Salt concentrations above 6-8% can inhibit biofilm formation by disrupting the bacterium's metabolic processes and damaging cell structures. In fact, at such concentrations, *L. monocytogenes* may experience cell death or growth stasis, and biofilm formation may be significantly reduced. High salt levels may also inhibit the production of EPS, thus preventing the bacteria from forming a stable biofilm matrix.

Pan and co-workers (2010) observed a synergistic effect of sodium chloride, glucose, and temperature on biofilm formation of *L. monocytogenes* serotype 1/2a and 4b strains. This study showed that the optimal conditions for biofilm formation by *L. monocytogenes* strains were 5% at 22.5 °C and 2% at 30 °C and 37 °C. Moreover, this study showed that none of the tested isolates used in this study were not able to form biofilm at low salt concentrations, which was unexpected based on previous laboratory experiments.

3.2.4.3 pH

According to studies, *L. monocytogenes* can survive in pH range from 4.2 to 9.5. Cole and co-workers (1990) reported that survival of *L. monocytogenes* is temperature dependent, when low pH values and high salt concentrations are used for inactivation. Moreover, in this study it was observed that *L. monocytogenes* survival was enhanced by low concentrations of salt even at very low pH values comparing with absence of additional salt.

Tresse and co-workers (2006) reported that adhesion capability of *L. monocytogenes* strains was reduced when cultivated at pH 5 compared to pH 7. This is attributed to the non-synthesis of flagellin under the acidic conditions, that proves that the conservation of food at pH 5 will delay the biofilm formation of *L. monocytogenes* in the food processing environments. However, some studies have shown that *Listeria* can adapt to acidic environments over time. Delaquis et al. (2014) reported that long-term exposure to acidic conditions could enhance the ability of *L. monocytogenes* to form biofilms in subsequent trials, possibly due to adaptive responses that increase the production of protective surface structures. Also, it was found that *L. monocytogenes* can survive even in the extreme pH, such as pH 3 for a period of time of 90 minutes (Davis et al. 1996).

Another study found out that *L. monocytogenes* biofilm forming ability is stimulated in vaginal fluid at different pH values, at pH 4.2, 5.5 and 6.5 respectively, whereas it is inhibited by the normal vaginal pH, at pH 3.8 to 4.5 (Borges et al. 2011). Similarly, to other studies (Skowron et al. 2019; Fan et al. 2020) that showed significantly inhibited biofilm formation of *L. monocytogenes* at pH 4 compared to neutral environment.

At pH levels above 8.0, Saito et al. (2019) found that biofilm formation by *L. monocytogenes* was reduced compared to those formed at neutral pH. This reduction in biofilm formation at high pH may be due to the decreased production of EPS or alterations in the bacteria's metabolism.

3.2.4.4 Microbial interactions

Bacteria in nature during interaction with other species can form structured multispecies biofilms. Biofilms can be defined as microbial communities involving multispecies associations with complex structures that have more ecological advantages comparing with the individual species present. Depending on the microbial species involved in the biofilm and the environmental conditions intracellular interaction of microorganisms within biofilms can be competitive, cooperative, or even neutral (Giaouris et al. 2015).

Biofilms of mixed cultures are complex communities formed by different microbial species that interact with each other including pathogenic and non-pathogenic species. Research has identified several microorganisms that can coexist in these biofilms, highlighting their interactions and

implications for health and industry. Some examples of microorganisms forming mixed biofilms are:

- *Listeria monocytogenes*: This foodborne pathogen often forms mixed biofilms with other bacteria, exhibiting increased resistance to disinfectants and antibiotics, posing significant risks in food safety (Chen et al. 2024).
- *Cronobacter sakazakii* and *Staphylococcus aureus*: In food processing environments, these bacteria can form hybrid biofilms, with *S. aureus* enhancing the biofilm-forming capabilities of *C. sakazakii* (Song et al. 2023).
- *Salmonella* Typhimurium and *E. coli* O157:H7: In meat processing environment, these pathogens formed biofilms with meat processing surface bacteria and showed that the presence of other species influences biofilm dynamics and pathogen persistence (Yang et al. 2023).
- *Oenococcus oeni* and *Saccharomyces cerevisiae*: In winemaking, these species formed mixed biofilms that improved fermentation efficiency and stress resistance, highlighting their practical application in the food industry (Palud et al. 2024).
- *Streptococcus suis* and *Actinobacillus pleuropneumoniae*: These two bacteria are known to co-infect the respiratory tract of swine, forming mixed biofilms that enhance drug resistance and complicate treatment efforts (Yi et al. 2022).
- *Bifidobacterium longum*, *Bacteroides ovatus*, *Enterococcus faecalis*, and *Lactobacillus gasseri*: These gut microbiota species have been shown to synergistically form robust mixed-species biofilms, improving their survival and metabolic activity (Xu et al. 2023) (Xu et al. 2022).

L. monocytogenes as a poor biofilm former comparing with other bacterial species, can have interspecies interactions with other bacteria (Kalmokoff et al. 2001). This attachment of *L. monocytogenes* cells in food processing surfaces previously colonized by other bacteria present a major risk for the food industry. The accompanying microbiota plays an important role on the number of adhered cells of *L. monocytogenes* that can be increased, decreased or unaltered in the mixed biofilm (Carpentier and Cerf 2011).

A decrease in *L. monocytogenes* cell numbers was observed in multispecies biofilms formed with *Staphylococcus xylosus* and *Pseudomonas fragi*, comparing with *L. monocytogenes* cell numbers

grown in monocultures. Also, it was observed that *L. monocytogenes* strains exhibit temperature-dependent adherence to stainless steel.

4. MATERIAL AND METHODS

4.1 Bacterial strains and serotypes

A total of 15 previously characterized and serotyped *Listeria* strains of 6 *Listeria* species isolated from various sources were included in this study (Table 2-3). The different bacterial strains were stored at - 80 °C in mixture of TS broth (TSB) and glycerol, which were then recovered on Brain Heart Infusion (BHI) Agar, cultivated at 37 °C for 24 hours and then streaked onto Trypto-Casein Soy Agar (TSA), cultivated at 37 °C for 24 hours.

Table 4. Non-*monocytogenes Listeria* species used in this study

Species	**Isolate ID**	**Origin**
L. innocua	CCM4030^T	cow brain
L. innocua	2885	unknown
L. seeligeri/welshimeri	292	unknown
L. welshimeri	CCM3971^T	decaying
L. ivanovii	204	vegetation
L. denitrificans	1157	unknown

CCM= Czech Collection of Microorganisms.

Table 5. *Listeria monocytogenes* strains used in this study

Notation	**Species**	**Isolate ID**	**Origin**
1	*L. monocytogenes*	NCAIM B1454	unknown
2	*L. monocytogenes*	3b T1	unknown
3	*L. monocytogenes*	11/4.12$t_0$3	isolated from cheese
4	*L. monocytogenes*	CCM 5576	guinea pig
5	*L. monocytogenes*	CCM 7202	spinal fluid of a child
6	*L. monocytogenes*	NCAIM B1966	guinea pig
7	*L. monocytogenes*	NCTC 10887	chinchilla
8	*L. monocytogenes*	NCTC 5105	human
9	*L. monocytogenes*	CCM 4699	sheep

NCAIM= National Collection of Agricultural and Industrial Microorganisms, Hungary.
NCTC= National Collection of Type Cultures.

4.2 Growth media and broth

4.2.1 Brain Heart Infusion (BHI) Agar

Brain Heart Infusion (BHI) Agar, as the most commonly used non-selective media for cultivation of *Listeria* species, was used in my experiment for the recovering of the frozen stock *Listeria* cultures. This agar was prepared by adding 37 g Brain Heart Infusion powder (Merck, Darmstadt, Germany) and 15 g bacteriological Agar to 1L of distilled water and sterilizing by autoclaving at 121 °C for 15 minutes.

4.2.2 Caso (TSA) Agar

Tryptone-Casein Soy Agar (TSA, Merck, Darmstadt, Germany) is a general-purpose medium that is usually used for growth of fastidious microorganisms, which tend to have high nutritional requirements, such as *Listeria.* To prepare Caso Agar, 15 g Tryptone-Casein Soy Broth powder (Merck, Darmstadt, Germany) and 15 g bacteriological agar were dissolved in 1L of distilled water. The media was sterilized by autoclaving at 121 °C for 15 minutes.

4.2.3 Tryptone-Casein Soy Broth

Tryptone Soy Broth (TSB, Merck, Darmstadt, Germany) is a general-purpose broth, commonly referred to as Casein Soya Bean or Soybean-Casein Digest Broth, that will support the growth of a wide range of micro-organisms including *Listeria*. To prepare TSB, 30 g of Tryptone-Casein Soy Broth powder was dissolved in 1L of distilled water and sterilized by autoclaving at 121^{o}C for 15 minutes.

4.2.4 Muller-Hinton Agar

Muller-Hinton Agar (MHA, Merck, Darmstadt, Germany) is a microbiological growth medium that enable the growth of different fastidious microorganisms, including *Listeria* species. In our experiment Muller-Hinton Agar was used for preparation of the overnight cultures, as one of the steps of the microtiter plate assay. The agar was prepared by adding 34 g of Muller-Hinton agar powder to1L of distilled water, sterilized by autoclaving at 121^{o}C for 15 minutes.

4.3 M9 Minimal Media

The minimal medium is a culture medium that contain the minimal amount of components for growth of the wild-type microorganisms, usually containing inorganic salts, a carbon source and water. The minimal media M9 was used in our study for testing the capability of biofilm formation of different *Listeria* strains. It was prepared by combining the following components and concentrations as presented in the table below (Table 6).

Table 6. Minimal Media M9 composition

Components	NH_4Cl	Na_2HPO_4	KH_2PO_4	NaCl	$MgSO_4$	$CaCl_2$	Glucose 20%
Concentration	1 g/L	6 g/L	3 g/L	0.5 g/L	2 mL/L	0.1 mL/L	10 mL/L

4.3.1 M9 Minimal Media preparation

M9 Minimal Media was prepared in two steps:

- Measurement of the following components: NH_4Cl, Na_2HPO_4, KH_2PO_4, NaCl, with the exact concentrations per 1L of distilled water as presented in Table 6., and then sterilization of the prepared solution by autoclaving at 121 °C for 15 minutes.

- Whereas solutions of $MgSO_4$ 1M, $CaCl_2$ 1M, and glucose 20 % with the above-mentioned concentrations (Table 6) were prepared and filter sterilized using a 25 mm diameter sterile syringe filter (FilterBio®) with a 0.22 μm pore size hydrophilic Polyethersulfone (PES) membrane, which were added separately in the autoclaved solution (Figure.2).

Figure 2. Filter sterilization of $MgSO_4$, $CaCl_2$, and glucose 20%.

4.4 Culture preparation

Fifteen *Listeria strains* were selected for this study (Table 4-5). These isolates were obtained from the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary and further maintained at the Department of Food Microbiology, Hygiene and Safety of the Hungarian University of Agriculture and Life Sciences. The cultures, which were derived from frozen stocks (− 80 °C), were cultivated on Tryptic Soy Agar plates and incubated at 37 °C for 24 hours (Figure. 3).

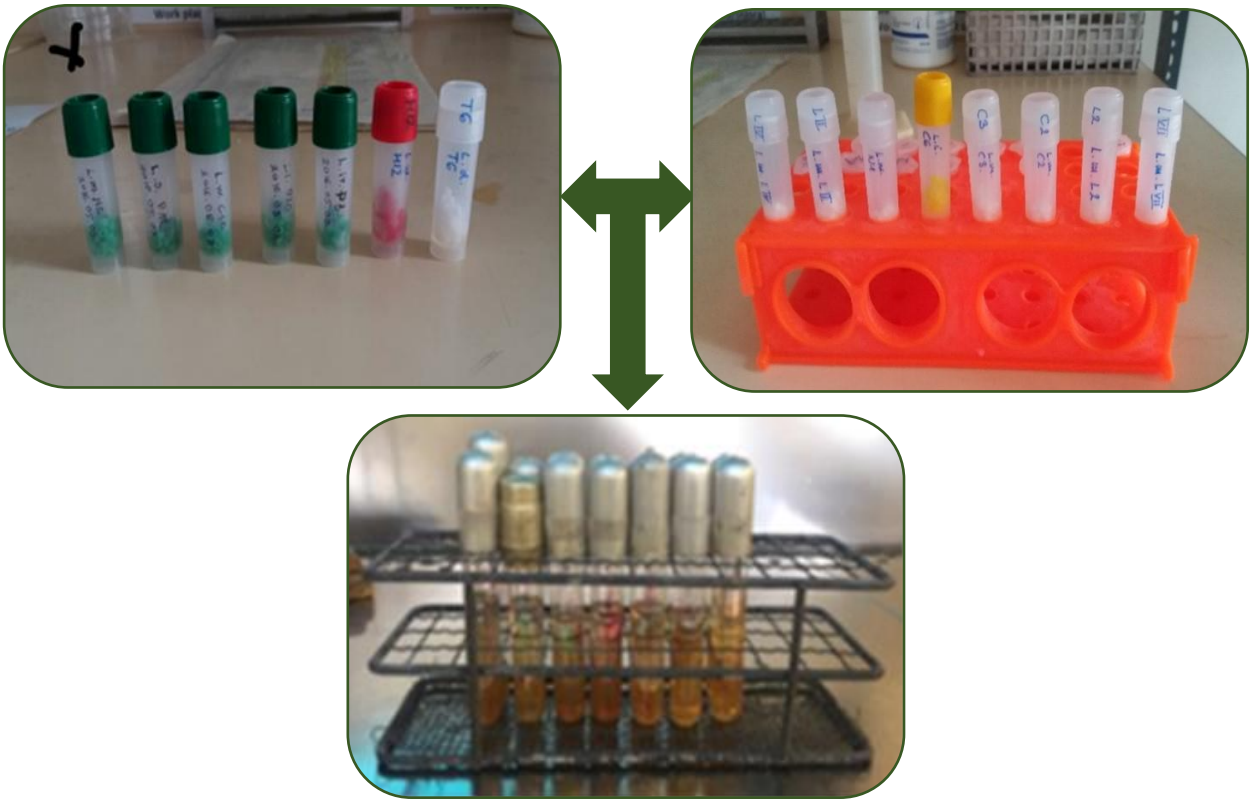


Figure 3. Culture preparation from frozen stocks.

4.5 The effect of pH, sodium chloride, and temperature on the biofilm formation of *Listeria* strains

4.5.1 Microtiter plate biofilm production assay

1. Fresh cultures were prepared in Trypto-Casein Soy Broth (TSB), cultivated at 37 °C for 24 hours. Biofilm formation was investigated using the crystal violet 0.4% (w/v) staining method by Mouwakeh (2018) with minor modifications. Laboratory-generated biofilms were grown in an ELISA titer plate with a final volume of 200 µL of M9 minimal media (pH6; 0 % NaCl) . Initial

cell-counts were adjusted to an optical density of 0.3 using a DEN-1B McFarland densitometer (Biosan) and then the ELISA titer plates were incubated at 37 °C for 48 hours.

a) In order to study the response of *Listeria* strains towards different stress factors, the composition of the M9 Minimal media was changed. Different concentrations of NaCl (0%, 5%, 10%, and 15%) in the composition of M9 Minimal media (pH6) were used in the case of the experiment of testing the biofilm ability of *Listeria* strains to NaCl as a stress condition, incubated at optimum temperature (37 °C). In case of analyzing the pH effect on the biofilm-forming ability of *Listeria* strains, different pH values (pH4, pH5, and pH6) of M9 Minimal (NaCl 0%) media were used by adjusting the pH with HCl 1N solution, incubated at 37 °C.

b) In order to study the effect of temperature on the biofilm formation of *Listeria monocytogenes* strains, experiments were carried out at different environmental temperatures (1 °C, 4 °C, 20 °C, and 37 °C) in M9 Minimal media incubated for 7 days at 1 °C and 4 °C; and 48 hours at 20 °C and 37 °C, when the pH6, and NaCl concentration was 0 %.

c) In the purpose of studying the effect of environmental (stress) conditions, experiments were carried out to analyze the effect of temperature (1 °C, 4 °C, 20 °C, and 37 °C), sodium chloride (0%, 5%, 7.5%, and 15%), and pH (4, 5, and 6) applied individually and in combination, on the growth and decline of *L. monocytogenes* in biofilm. For these experiments, the plates were incubated for 7 days at (1 °C and 4 °C), and for 48 hours at (20 °C and 37 °C).

2. After the incubation period, the supernatant from the wells of the plate was discarded to a bin with disinfectant, by turning the plates upside down. Then each well was washed (3x) with Phosphate-buffered-saline (PBS) solution (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 800 mL distilled water; pH adjusted to 7.4 with HCl), and the supernatant was discarded. In the last washing step when finished, the plate was taped in paper to remove the exciding PBS and the plates were dried for 15 min in the flow hood.

3. After that, the formed biomass was stained by adding 200 μL of Crystal Violet 0.4% (w/v) solution to each well, and the plates were incubated at room temperature for 15 min. Then the Crystal Violet was discarded. After that a second repeat of washing procedure with phosphate-buffered-saline (PBS) was applied following the same procedure as in the first washing step. Then the plates were dried for 15 min in the flow hood. Finally, in the last step 200 μL of acetic acid 33% (v/v) were added to each well, and the optical density were measured using a microtiter plate

reader (Multiscan Ascent, ThermoLab System) at 595 nm (OD_{595}). Multiskan Ascent instrument (Figure 4) was controlled through a computer using the Ascent Software Version 2.6 (ThermoLabsystems).

The average values and standard deviations of the optical density at 595 nm from the three replicates of each treatment were calculated for each *Listeria* strain. The standard deviations of the means were represented by error bars in the graphs.

Figure 4. Multiskan Ascent instrument.

4.6 Effect of sub-lethal temperature on the cells of *L. monocytogenes* biofilms

Nine *Listeria monocytogenes* isolates (NCAIMB1454, 3b T1, 11/4.12$t_0$3, CCM5576, CCM7202, NCAIM B1966, NCTC 10887, NCTC 5105, CCM 4699) were selected for this experiment.

In order to study the behavior of *Listeria monocytogenes* biofilms when submitted to the sub-lethal temperature, we used the same protocol applied for the determination of the effect of pH and sodium chloride. Shortly, fresh cultures were prepared in Trypto-Casein Soy Broth (TSB), cultivated at 37 °C for 24 hours. Biofilm formation was investigated using the crystal violet 0.4% (w/v) staining method. Laboratory-generated biofilms were grown in an ELISA titer plate with a final volume of 200 µL of M9 minimal media. Initial cell-counts were adjusted to an optical density of 0.3 using a McFarland densitometer (Biosan). In order to study the behavior of *Listeria monocytogenes* biofilm cells when submitted to stress conditions such as the sub-lethal

temperature, after creating the biofilms and before reading the optical density, the cultures were placed in a thermal bath at 50 °C for 60 min.

Finally, in the last step, the optical density at 595 nm from the three biofilm assays was measured using a microtiter plate reader (Multiscan Ascent, ThermoLab System). Multiskan Ascent instrument was controlled through a computer using the Ascent Software Version 2.6 (ThermoLabsystems) and the heat-treated and non-heat-treated strains were compared regarding their biofilm formation ability.

4.7 The effectiveness of different antimicrobials on *L. monocytogenes* biofilms formed on the surface of lettuce leaves

The effectiveness of different antimicrobials on *L. monocytogenes* biofilms formed on the surface of lettuce leaves is an important area of food safety research, as this pathogen can cause serious foodborne illness. Choosing the right antimicrobial is essential, and different types of antimicrobials have different levels of effectiveness.

For this experiment, two strains (selected based on previous experiments) of ***L. monocytogenes*** were used:

- NCAIM B1966: strain with strong biofilm forming capacity.
- CCM 5576: strain with weak biofilm forming capacity.

Experiments were performed to analyse the effect of some antimicrobials (industrial/natural) (chlorine, acetic acid, balsamic vinegar, white vinegar, and lactic acid) against the growth of these two strains on the surface of lettuce leaves.

Figure 5. Balsamic vinegar and white vinegar used in this experiment.

1) The experimental media (TSB) was inoculated with 1% (v/v) of an overnight *Listeria* culture (NCAIM B1966 or CCM 5576), thus the initial cell concentration was 10^6 CFU/ml. The lettuce leaves (1x1cm squares) were disinfected with alcohol (70%), and then they were dipped into the inoculated media and incubated for 1 hour at room temperature.

2) After 1 hour of incubation, both sides of the lettuce leaves were treated with one of the following antimicrobial compounds:

- chlorine (for 5 minutes),
- acetic acid or lactic acid (for 10min),
- undiluted balsamic vinegar or white vinegar (for 15min).

3) After that, the lettuce leaves were rinsed with 10 ml of sterile distilled water to eliminate reversibly attached bacteria. This was followed by mechanical removal of the attached cells using a vortex and maintained the vortexing for 50 seconds (Figure 6).

4) The biofilm formation was monitored by determining the attached cell number after 24h, and 48h of incubation. Then, serial ten-fold dilutions were done, and the cell number was determined by spread plate technique.

The average values and standard deviations of the three repetitions were calculated for each *Listeria* strains. The standard deviations of the means were represented by error bars in the graphs.

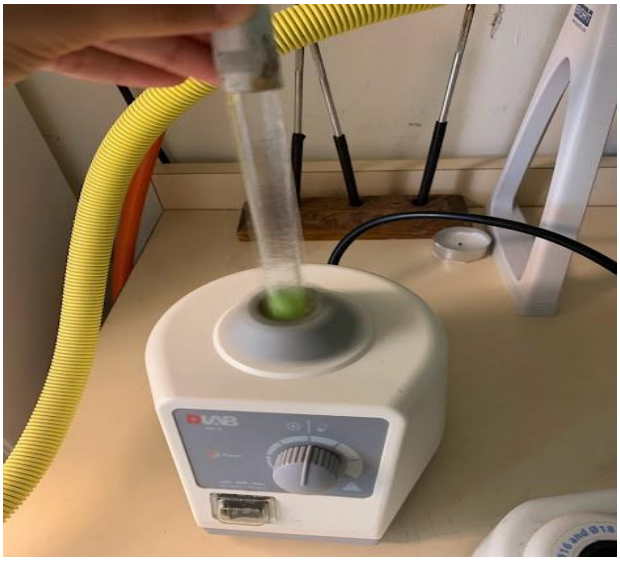


Figure 6. Mechanical removal of the attached cells

4.8 Data analyses

For data analyses, statistical software IBM SPSS Statistics (version 27) was used. One-factor analysis of variance (ANOVA) was used to determine statistically significant differences between the *L. monocytogenes* strains' biofilm formation ability after incubation at optimum environment (37 °C, pH=6, and 0 % NaCl).

Discriminant analysis was applied to conduct a multivariate analysis of variance test of the hypothesis that the 9 *L. monocytogenes* strains could be classified into 3 groups (weak, moderate, or strong biofilm former). The assumptions were tested as homogeneity of variances was not violated based on Levene Statistic=1.58; p=0.20, based on the Shapiro-Wilk's tests (test values>0.874; p>0.30) the normality assumption is satisfied across the replicates and in the dataset no outlier was detected using Z score values after standardization (values<2).

For further analysis, OD values at optimum environment were used as a positive controls and OD values recorded after the incubation at the most adverse tested environment (1 °C, pH=4, and 15 % NaCl) inferred as negative controls.

In further analyses, the average of the three measurements was taken and OD values based on the positive (OD_{max} – average of positive control values) and negative (OD_{min} – average of negative control values) controls were normalized. In that way the biofilm formation capacity (%) could be expressed (Eq. 1) as:

$$Biofilm\ formation\ capacity\ (\%) = \frac{OD_x - OD_{min}}{OD_{max} - OD_{min}} \quad (1)$$

Using the biofilm formation capacity (%) values, the responses of *L. monocytogenes* strains classified into different groups could be compared.

The significances of the differences between the groups were verified by one-way ANOVA, and the mean comparison were performed by Tukey's test using Past program. The results were considered significant when $P \leq 0.05$.

5. RESULTS AND DISCUSSION

5.1 Biofilm Forming Ability of non-*monocytogenes Listeria* spp strains

Based on the original biofilm-forming ability of different *Listeria* spp. strains after 48-hour incubation at temperature 37 °C when pH=6, and NaCl concentration 0%, which results are shown in Figure 7.

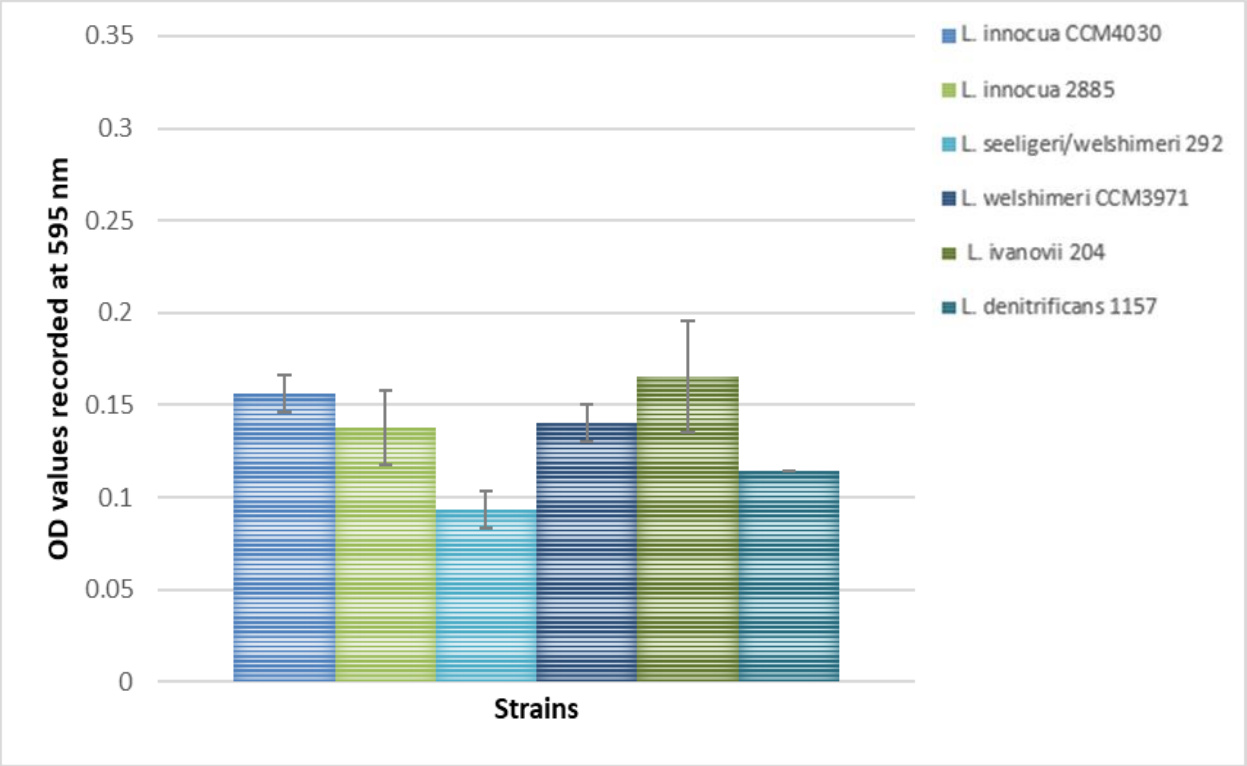


Figure 7. Biofilm formation of *Listeria* spp strains at optimum environment (37 °C, pH=6, and 0 % NaCl) based on the measured optical density values at 595 nm. Errors bars represent the standard deviations of the means (Hasani et al. 2021).

According to Figure 7, the pathogenic *Listeria* strain *L. ivanovii* 204 and the non-pathogenic *L. innocua* CCM4030 formed the biggest amounts of biofilm (OD595 > 0.150) when incubated in M9 Minimal Media at temperature 37 °C. The other non-pathogenic strains formed less biofilm amounts, with *L. seeligeri/welshimeri* 292 being the weakest biofilm forming strain (OD_{595} > 0.1).

5.2 The effect of NaCl concentration and pH on biofilm formation of different *Listeria* spp strains

5.2.1 Effect of NaCl concentration on the biofilm formation of *Listeria* spp strains

The biofilm formation of different *Listeria* strains under different NaCl concentrations can be seen in the Figure 8. According to our results, *Listeria ivanovii 204* formed larger quantities of biofilms than the other non-pathogenic *Listeria* strains when incubated in M9 Minimal Media at 0.05% NaCl concentration at temperature 37 °C.

From Figure 8, the OD_{595} of the *Listeria ivanovii* 204 incubated in M9 Minimal Media at 37 °C with increasing NaCl concentration from 0.05 to 10% dropped from 0.145 to 0.090, probably because the bacteria growth was inhibited. When NaCl concentration is adjusted to 15%, biofilm formation of the *Listeria ivanovii* 204 did not decrease any further.
With the exception of *Listeria seeligeri/welshimeri* 292, all the other strains in the study showed no significant differences ($P \leq 0.05$) of OD_{595} values at 10 and 15% NaCl concentrations.

Similar patterns of biofilm formation (OD_{595}) were observed in *Listeria innocua* CCM4030, *Listeria innocua* 2885, and *Listeria seeligeri/welshimeri* 292 when grown on 5%–15% NaCl concentrations. A continuously decreasing trend of OD_{595} was detected for all the other strains, indicating that more biofilm was formed at 5% than at 10 and 15% NaCl concentrations. From these results, it can be concluded that the higher concentrations of NaCl did not present stress conditions that enhanced biofilm formation of non-pathogenic *Listeria* strains. Similar results were obtained when biofilm formation of *Listeria monocytogenes* strains were studied applying different NaCl concentrations (Pan et al. 2010; Xu et al. 2010).

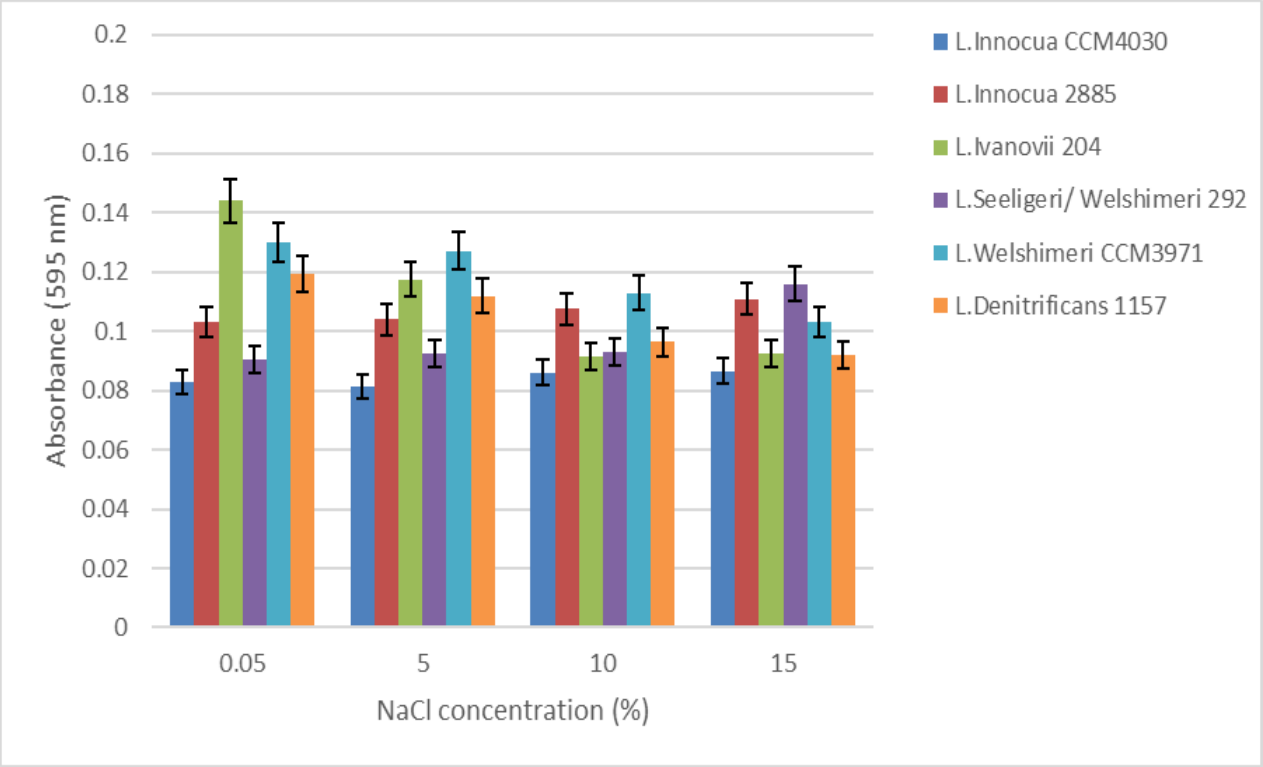


Figure 8. Biofilm formation (OD595) comparison between the studied *Listeria* strains following 72 h incubation at 37 °C under different NaCl concentrations (0.05, 5, 10, and 15%) using M9 Minimal Media (Hasani et al. 2021).

5.2.2 Effect of pH on the biofilm formation of *Listeria* spp strains

From Figure 9, all the *Listeria* strains incubated in M9 Minimal Media at 37 °C at different pH values (4, 5, and 6) showed a continuously decreasing trend of OD_{595}, except *Listeria ivanovii* 204. Therefore, more biofilm amounts were detected at pH 6 than at pH 4 and 5, respectively, which proves the inhibition effect of acidic conditions for the biofilm formation of *Listeria* strains tested.

The lowest amounts of biofilms were observed at pH 5 compared to pH 6 in M9 Minimal Media for all the strains ($P \leq 0.05$). At pH 4, *Listeria ivanovii* 204 and *Listeria denitrificans* 1157 were found to produce the most biofilm thus showing resistance to low pH. The most sensitive strain at pH 4 was *Listeria welshimeri* CCM3971 that showed weak biofilm formation. At pH 5, the lowest amounts of biofilm formed were observed from *Listeria ivanovi* 204 and *Listeria innocua* CCM4030, these were the weakest biofilms forming strains.

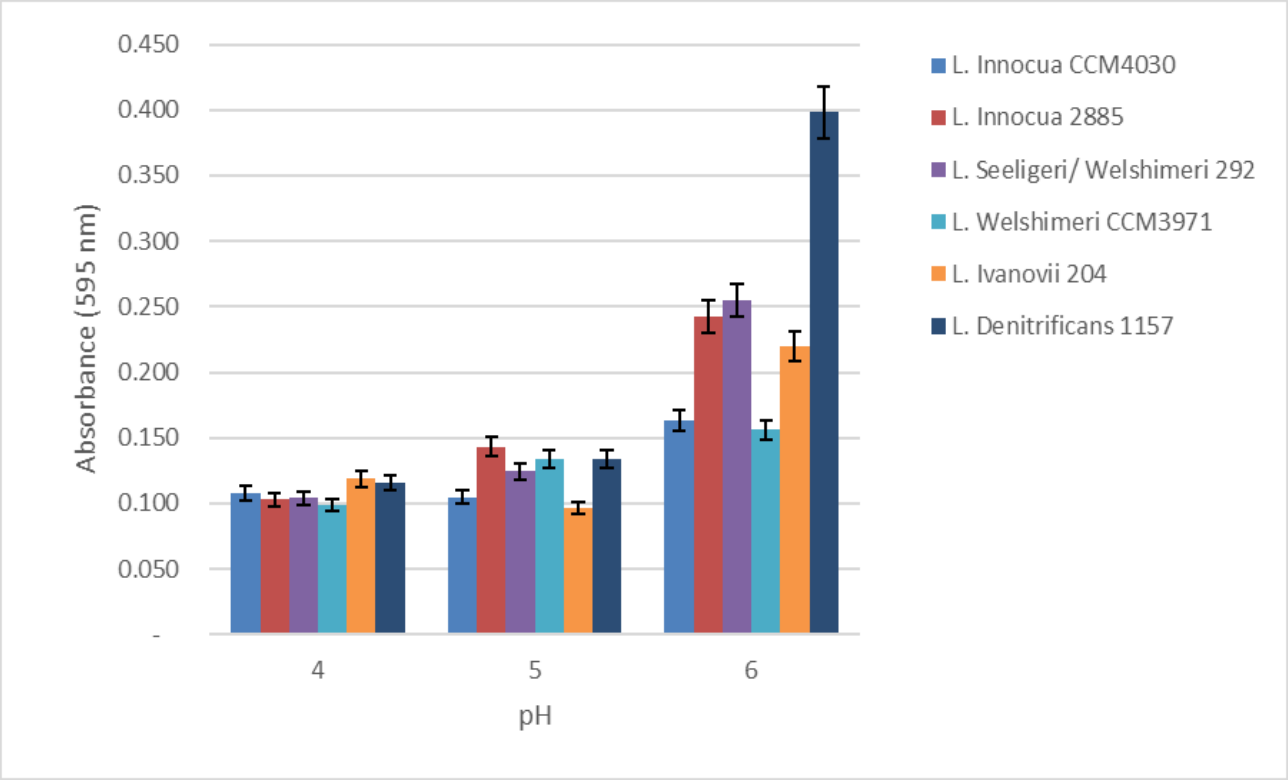


Figure 9. Comparison of biofilm formation (OD_{595}) among the studied *Listeria* strains following 72 h incubation at 37 °C under different pH values (4, 5, and 6) using M9 Minimal Media (Hasani et al. 2021).

5.3 The original Biofilm Forming Ability of *L. monocytogenes* strains - Positive control

The preliminary grouping was carried out based on the measured OD values (which are proportional to the biofilm-forming capacities) after 48-hour incubation at optimum environment: 37 °C; pH=6 and 0 % NaCl. The results of 9 strains could be divided into 3 groups (Figure 10): weak capacity <OD=0.2; moderate capacity 0.2 ≤OD≤ 0.25; strong capacity strains OD >0.25 (Table 7).

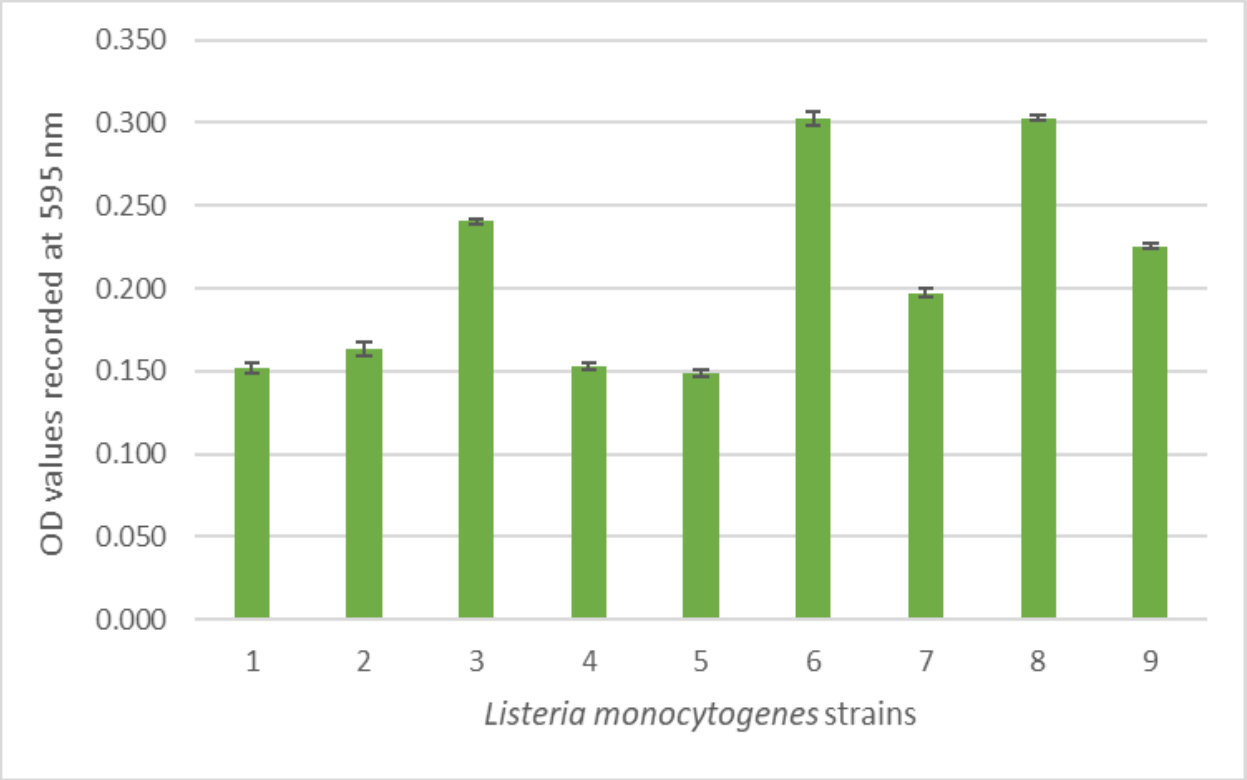


Figure 10. Biofilm formation of *L. monocytogenes* strains at optimum environment (37 °C, pH=6, and 0 % NaCl) based on the measured optical density values at 595 nm. Errors bars represent the standard deviations of the means.

As it can be seen in Figure 10, the optical density range of biofilm formation of the investigated *Listeria monocytogenes* strains was between 0,148 and 0,31. Comparing these values with the OD values of the non-*monocytogenes* strains, it can be seen that many *monocytogenes* strains reached much higher OD values. The highest optical density value achieved among non-*monocytogenes* strains was only slightly higher than the lowest value achieved by *Listeria monocytogenes* strains at optimal temperature. Based on the grouping of *Listeria monocytogenes* strains on the OD values, we can assume that all the investigated non-*monocytogenes* strains can be considered as weak capacity strains (<OD=0.2).

Table 7. Preliminary grouping of the *Listeria monocytogenes* strains.

Group	Biofilm formation ability	Strain	Notation
1	Weak	NCAIM B1454 3b T1 CCM5576 CCM7202	1 2 4 5
2	Moderate	11/4.12t03 10887 ½ a CCM 4699	3 7 9
3	Strong	NCAIM B01966T 5105 3a	6 8

Discriminant analysis

One-factor ANOVA showed a significant difference between the groups ($F=1696.03$, $p=2.76*10^{-24}$ value, $F_{crit}= 2.51$). Discriminant analysis was used to test our hypotheses regarding the strain's classification. The multivariate statistical analysis was used as a follow-up analysis to estimate the differences between the tested groups.

The Chi-square test was significant (Wilks $\lambda = 0.015$, Chi-square = 21.1, Canonical correlation =0.998, $p=0.002$), confirming our hypothesis. The classification of strains was based on the Mahalanobis distances to the group centroids which was highly successful: 100% of the cases were correctly reclassified into the 3 groups.

Based on these OD values measured at optimal conditions, discriminant analysis also proved that the strains can be divided into 3 groups in term of their biofilm-forming ability, which results are shown in Figure 11.

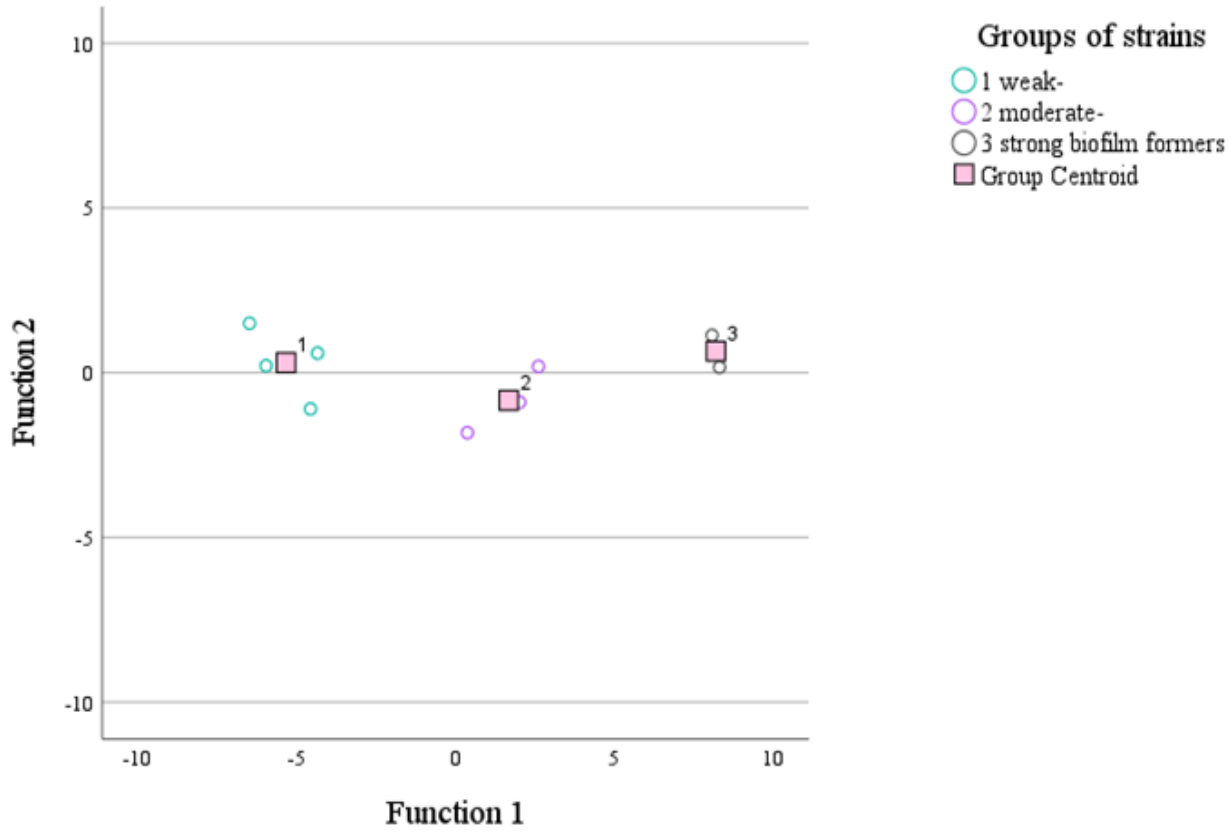


Figure 11. Canonical discriminant functions are used for the classification of the strains, based on their biofilm formation ability following incubation at optimum conditions.

5.4 The effect of temperature, NaCl concentration and pH on biofilm formation of different *Listeria monocytogenes* strains

Biofilm formation ability of *Listeria* strains was evaluated by optical density measurements determining the OD values at 595 nm in microtiter plates. The comparison of biofilm formation capacity (%) of different *Listeria monocytogenes* strains was investigated using the normalized OD values. The effect of temperature, NaCl and pH were tested separately and the biofilm formation capacity (%) was assumed 100 % for each strain at optimum conditions and 0 % at the most adverse incubation period as negative control. Studying these results, the responses of each bacterial strains could be compared to see the difference between these treatments.

5.4.1 The effect of temperature on the biofilm formation

The effect of temperature was tested at 1, 4, 20 and 37 °C, respectively, while the NaCl concentration and pH remained the same (0 mg/L and pH6). The average of optical density recorded at 595 nm are shown in Figure 12, and then the normalized capacity of the strains was calculated (Figure 13).

It can also be observed in Figure 12, that the optical density range of biofilm formation of the investigated *Listeria monocytogenes* strains at different temperature was between 0,1 and 0,31, and for the weaker biofilm-forming strains, the effect of temperature reduction was not as strong as for the stronger biofilm-forming strains.

At optimal conditions (at 37 °C), the ability of strains to form biofilm was considered to be 100%. Investigating the temperature effect, we found that there was no significant difference between 1 and 4 degrees ($p > 0.05$) (Figure 13).

At 20 °C the biofilm forming ability of the strains decreased with an average of 16 %. At 1 °C and 4 °C, the rate of inhibition of growth of *L. monocytogenes* strains was higher as it was expected, but even at 1 °C groups were formed. A consistent decrease in the population of *L. monocytogenes* strains was observed after 7 days of incubation at low temperature. These observations indicate that the viability of *L. monocytogenes* strains is influenced by refrigeration. Which is in consensus with the results of other researchers (Djordjevic et al. 2002; Fan et al. 2020). Although biofilm formation was weaker at refrigerated temperatures, but the investigated strains were able to form biofilm at these low temperatures as well, which poses a significant food safety risk. The ability of *L. monocytogenes* to form biofilms at low temperatures increases the potential for cross-contamination during food production.

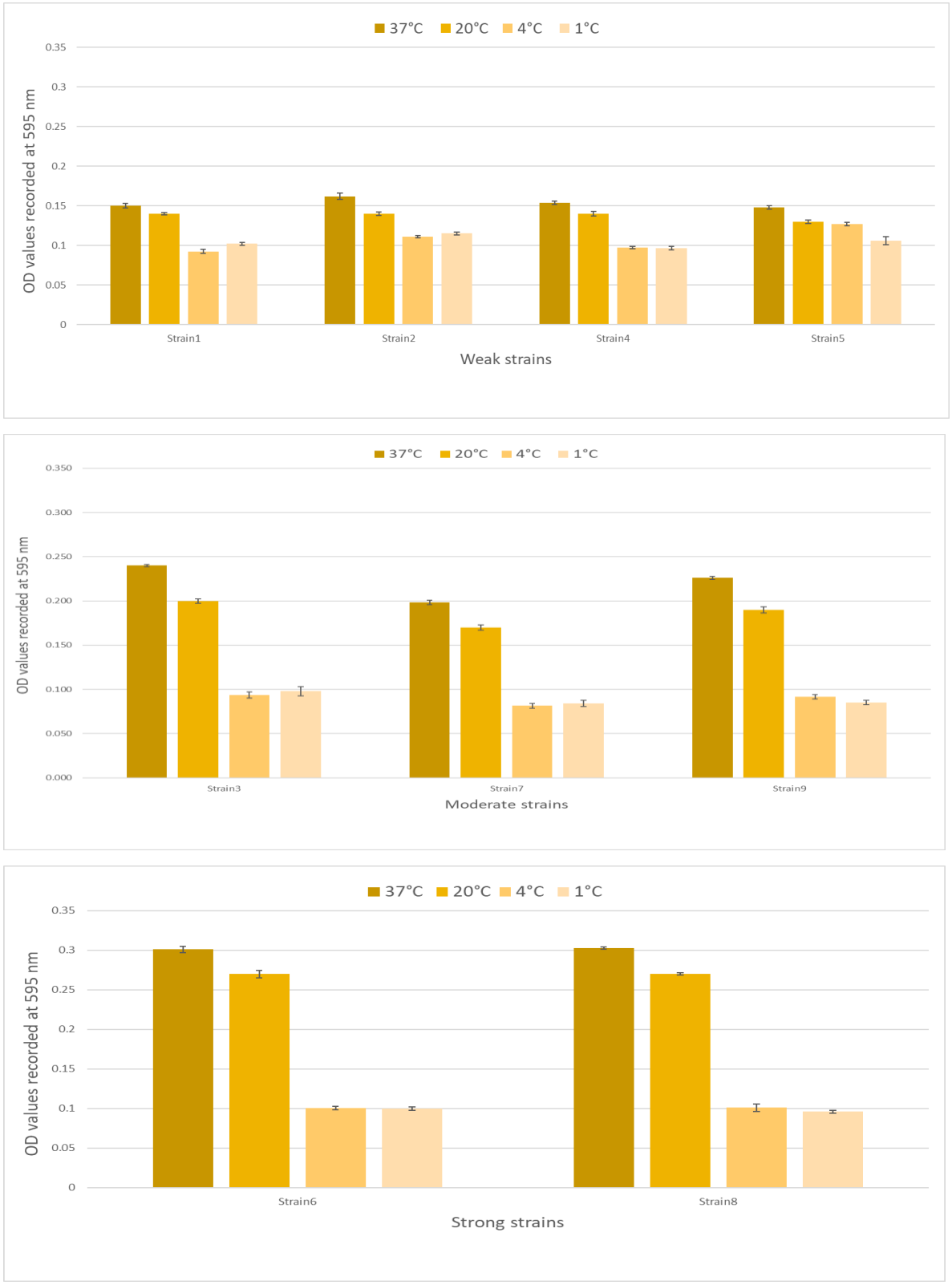


Figure 12. Biofilm formation of *L. monocytogenes* strains at 1°C, 4°C, 20°C, and 37 °C (pH=6, and 0 % NaCl) based on the measured optical density values at 595 nm. Error bars represent the standard deviations of the means, from three individual measurements.

For biofilm formation a certain number of cells are necessary, and the number of cells depends on the initial attachment (Dickson and Daniels, 1991). It was proved that increasing attachment temperature increases the number of adhered cells (Mai and Conner, 2007).

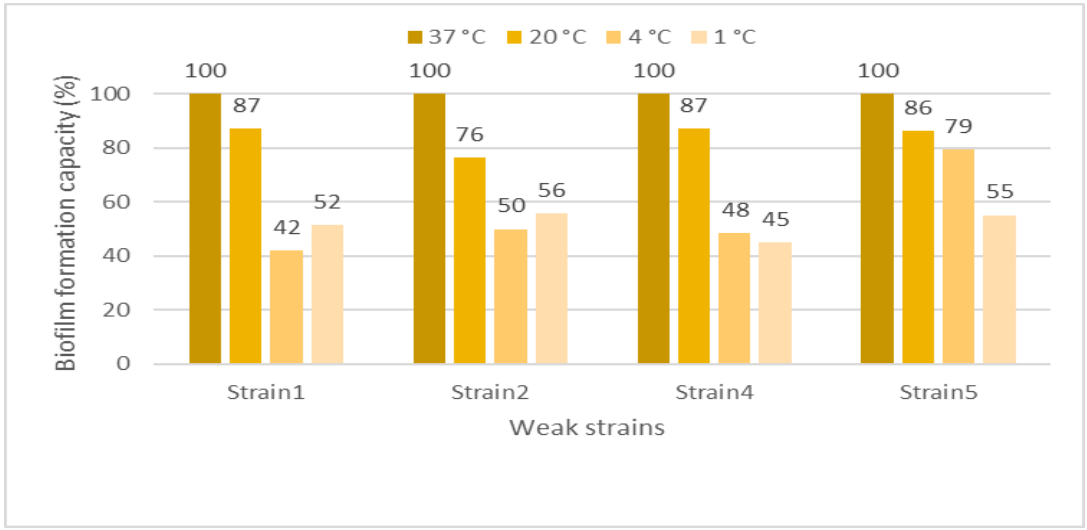
Temperature is a very important parameter because it can influence biofilm formation in various ways. Garrett and co-workers (2008) proved that the reaction rate decreases away from the optimum growth temperature, resulting in slower bacterial growth and thus less biofilm formation.

Higher temperatures may have a positive effect on biofilm formation by improving the growth of *L. monocytogenes* at the optimum growth temperature, and it effects also on hydrophobicity. Fan and co-workers (2020) found that hydrophobicity positively correlated with biofilm formation, and hydrophobicity increased with increasing growth temperature (Chavant et al. 2004; Di Bonaventura et al. 2008).

Villain-Simonnet and co-workers (2000) demonstrated that the temperature also affects the viscosity of the polysaccharides in bacterial EPS which also influences biofilm formation. It was also stated that the appendages (e.g. flagella, pili) on the cell surface of bacteria that help them adhere to surfaces are dependent on temperature (Garrett et al. 2008).

Our results showed that at lower temperatures the biofilm forming capacity of the strains decreased gradually, the strain's ability in Group1 (weak biofilm formers) decreased with an average of 48%, in Group2 (moderate biofilm formers) with an average of 76%, and in Group3 (strong biofilm formers) with an average of 80 %. So, we can conclude that the strains which had originally lower biofilm-forming capacity were less sensitive to the temperature effect. In Group 1, strain CCM7202 (strain 5) was more resistant to temperature effect, at 4 °C its biofilm-forming ability was still 79 %.

A



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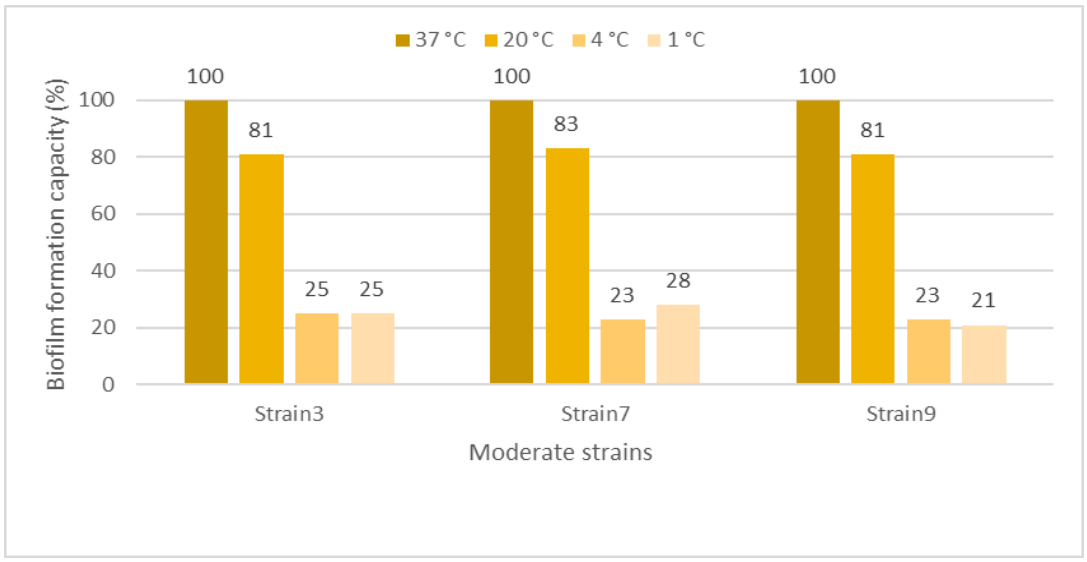

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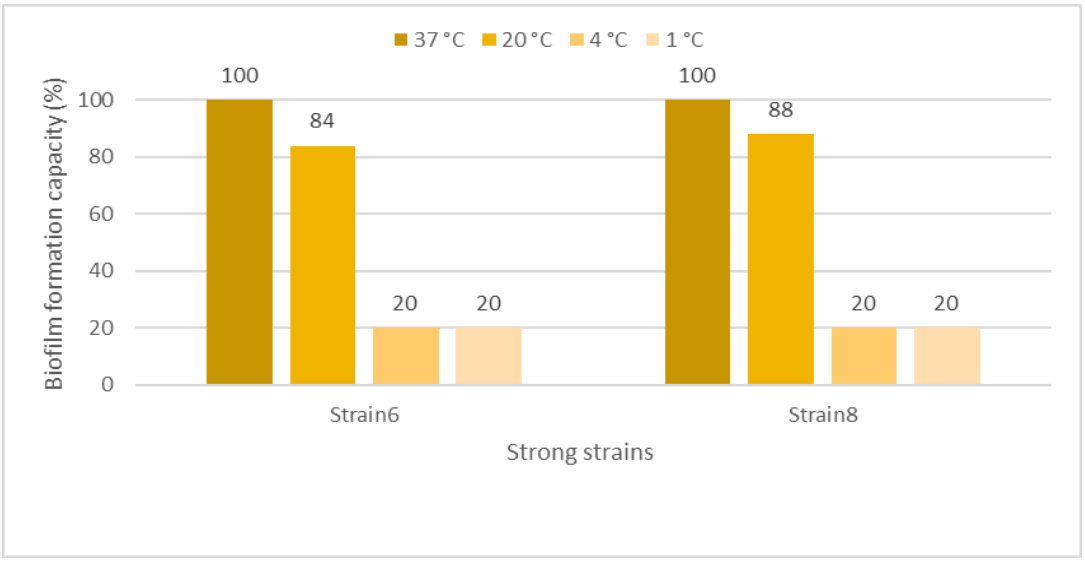


Figure 13. The effect of different incubation temperatures on biofilm formation capacity of *L. monocytogenes* strains incubated for 7 days at 1 °C and 4 °C, and 48 hours at 20 °C and 37 °C, (pH=6, and NaCl concentration 0 %).

5.4.2 Effect of NaCl on the biofilm formation

The effect of sodium chloride was examined by adding 0, 5, 7.5, and 15 % to the media, while the incubation temperature was 37 °C and pH was set to 6. From the optical density recorded at 595 nm (Figure 14), we calculated the normalized values (Figure 15).

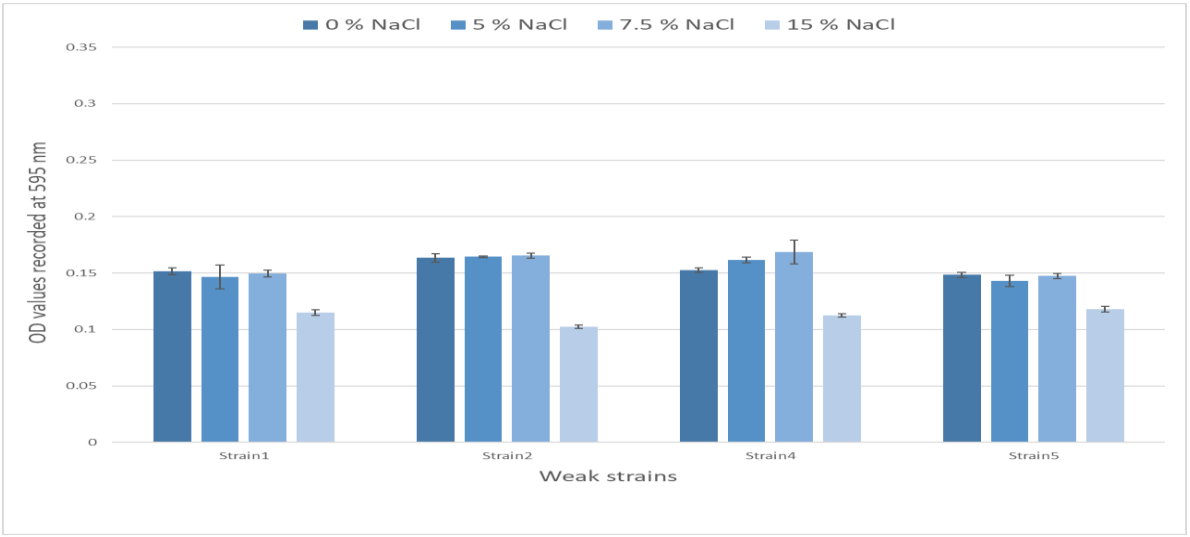
The effect of osmolarity was examined as it was shown that in some bacteria e.g. in *Shigella boydii* and *Listeria monocytogenes*, biofilm formation was found to be associated with high osmolarity (Zhao et al. 2017). It was observed that biofilm formation of *S. aureus*, which is similarly salt tolerant as *Listeria monocytogenes*, increased with increasing salt concentration (e.g. increased from 4% to 6%) (Silva et al. 2022). While high osmolarity was proved to be inhibitory for biofilm formation of various bacteria (Kamjumphol et al. 2013) such as *Pseudomonas fluorescens*.

Figure 14 shows that similarly to the temperature stress, the optical density range of biofilm formation of the investigated *Listeria monocytogenes* strains at different NaCl concentration was between 0,1 and 0,31. As with the effect of temperature, it can also be observed for the weaker biofilm-forming strains, that the effect of NaCl concentration increase was not as significant as for the stronger biofilm-forming strains.

Analysing the normalized values (Figure 15), we found that there was no significant difference between the responses of the weak biofilm former strains ($F=0.68$, $p=0.53$, $F_{crit}=4.26$) between 0 and 7.5 % NaCl, and they do not have a significant effect on the inhibition of *L. monocytogenes* strains. However, with 15% NaCl, decreasing amounts of biofilms could be observed. Similar decreasing patterns of biofilm formation were observed by Pan and co-workers (2010) in *L. monocytogenes* serotype 1/2a and 4b after increasing the NaCl concentration from 0.5 to 7% at a temperature of 37 °C.

With 15% NaCl, we found that the strains biofilm formation ability in Group1 decreased with an average of 42%, in Group2 with an average of 49%, and in Group3 with an average of 77%, except for strain CCM 4699 (strain 9) there was just 26% of decreasing, showing resistance and adaptation to high concentration of sodium chloride. In Group 3, strain NCAIM B01966T (strain 6) was more sensitive to sodium chloride treatment, its biofilm-forming ability was 20%. Additionally, we can conclude that the strains which had originally higher biofilm-forming capacity were more sensitive to the sodium chloride treatment.

A



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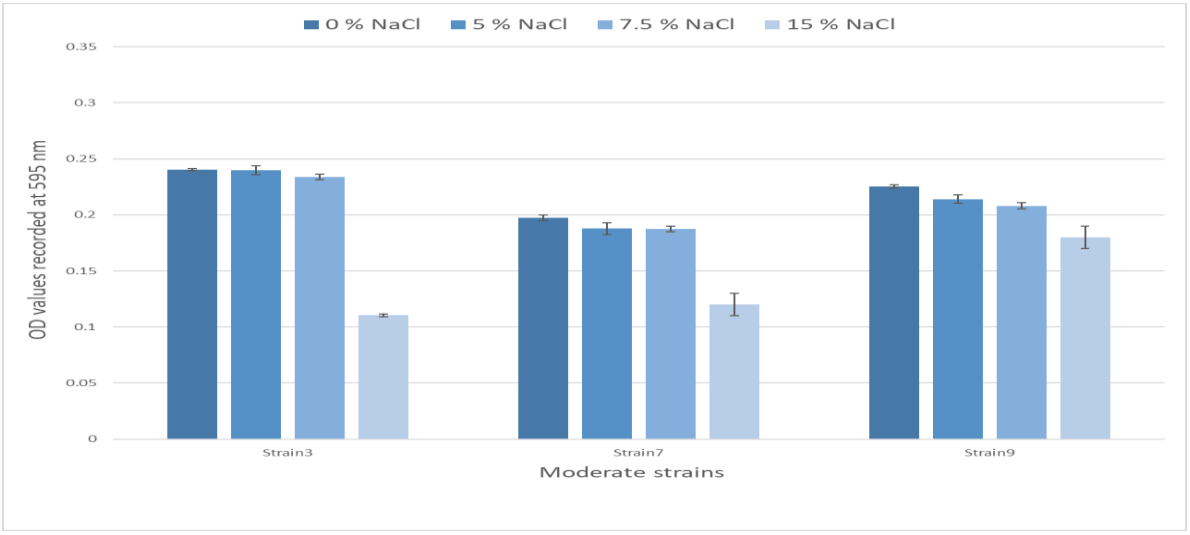

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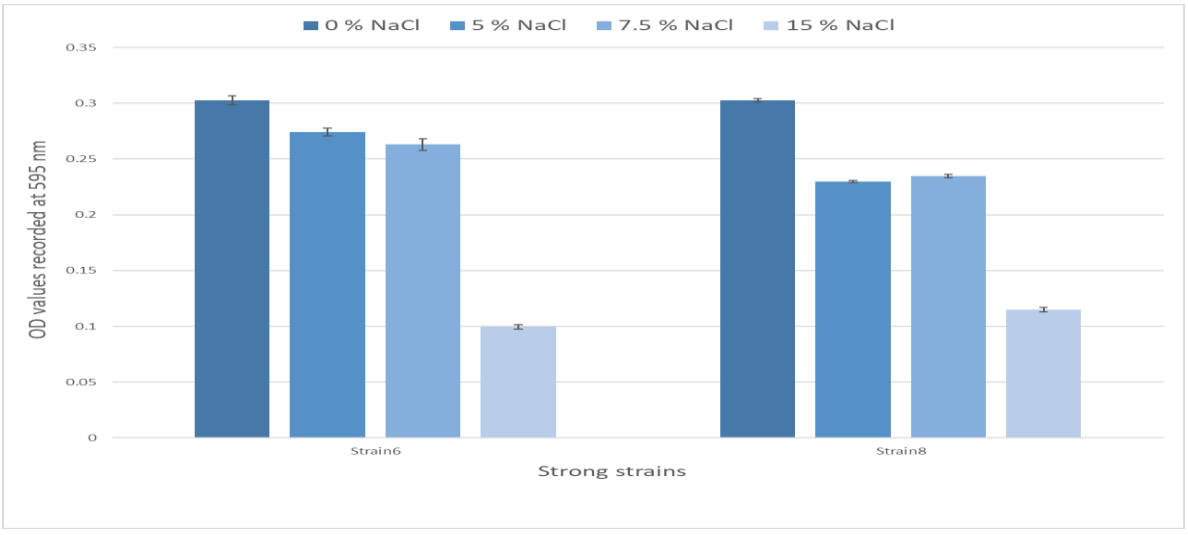
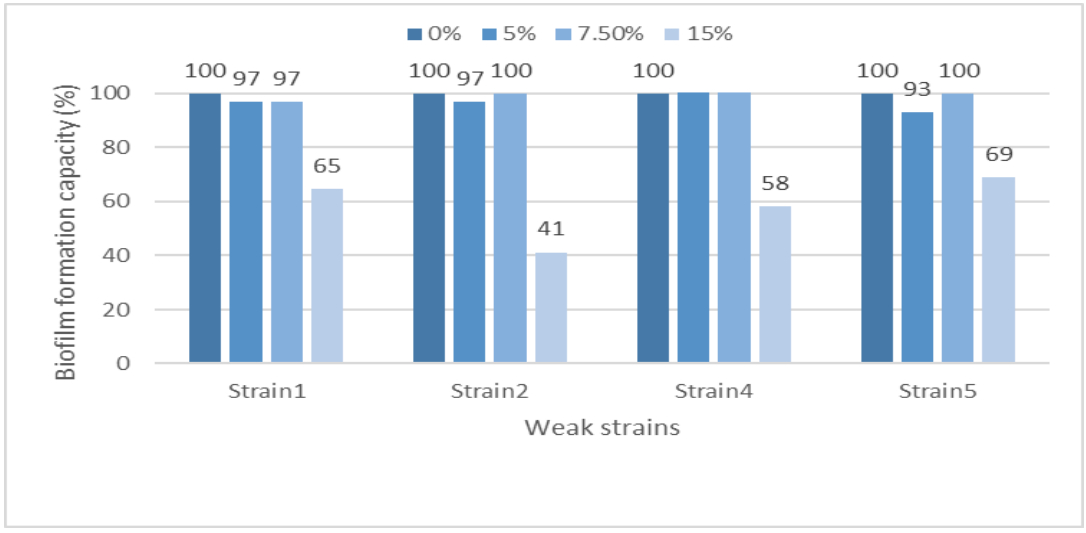


Figure 14. Biofilm formation of *L. monocytogenes* strains applying different NaCl concentrations (0 %,5%, 7,5% and 15%) at 37 °C, when pH=6) based on the measured optical density values at 595 nm. Error bars represent the standard deviations of the means, from three individual measurements.

A



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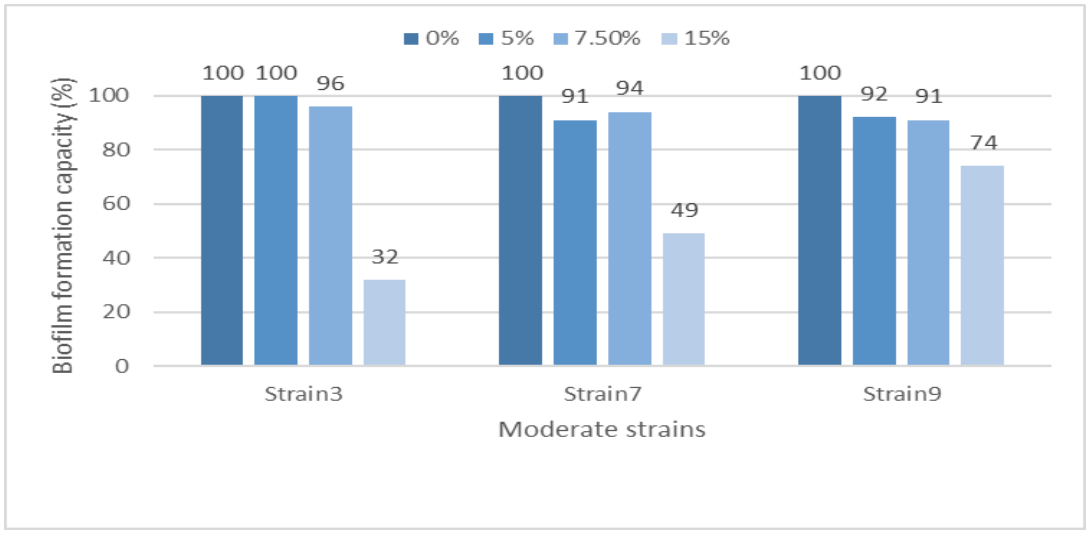

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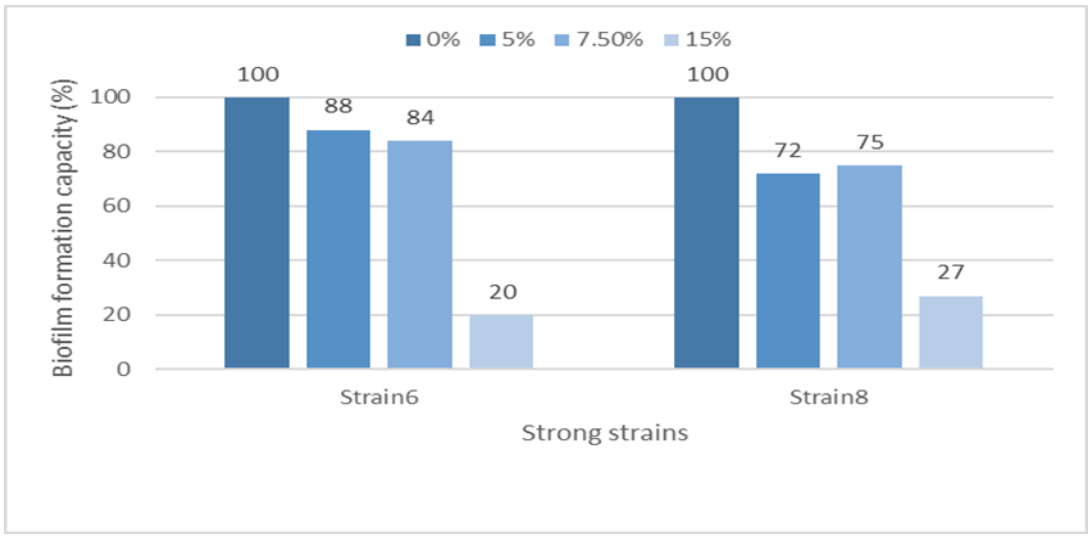


Figure 15. The effect of different NaCl concentrations on biofilm formation capacity of *L. monocytogenes* strains incubated at 37 °C, pH=6, and at NaCl concentrations 0 %,5%, 7,5%, and 15%.

5.4.3 Effect of pH on the biofilm formation

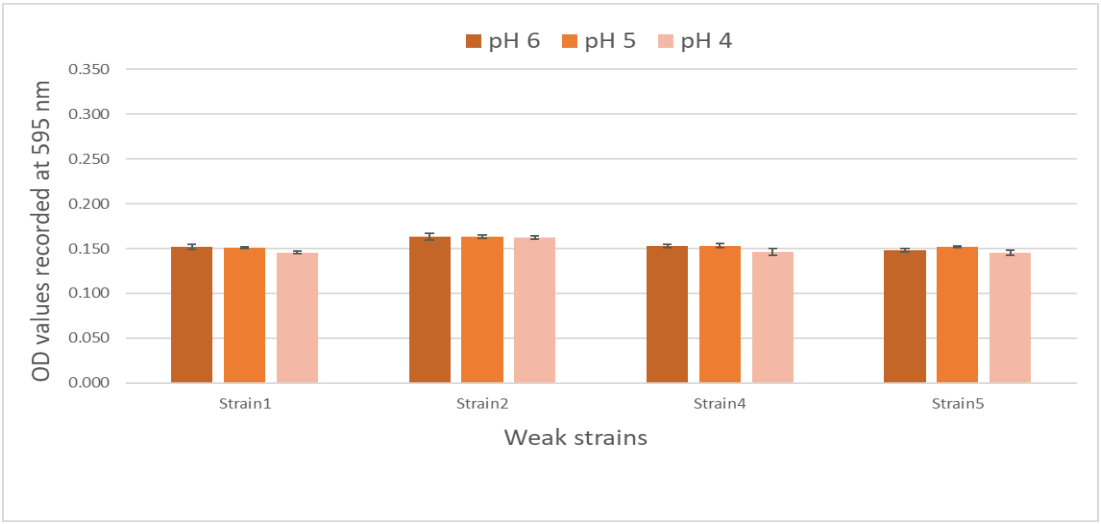
Regarding the pH effect, it was tested by adjusting the pH of M9 minimal media to 4, 5, and 6 when the incubation temperature was 37 °C and NaCl concentration 0%. It could be noticed that all tested *L. monocytogenes* strains formed biofilms at pH of 4, 5, and 6 (Figure 16) that is in the survival range of the strain, which has been reported to grow between pH 4.2 to pH 9,5 (Food Safety Authority of Ireland, 2005).

Figure 16 shows that the pH range tested had the least effect on the biofilm formation of the strains tested (the optical density range of biofilm formation of the investigated *Listeria monocytogenes* strains at different pH values was between 0,15 and 0,31). However, a different response between groups was also observed for the effect of pH, that is, similarly to the effect of temperature and NaCl, a decrease in pH had less effect on biofilm formation for the weaker biofilm-forming strains than for the stronger biofilm-forming strains.

Based on the normalized data (Figure 17), we found that, even pH 4 had no effect on the inhibition of *L. monocytogenes* of Group1 and 2, the ability of the strain in Group1 decreased with an average of 5% and in Group2 with an average of 6% . At pH values higher than 4, *L. monocytogenes* NCAIM B01966T (strain 6) and 5105 3a (strain 8) formed higher biofilm amounts showing that they prefer to grow and form biofilms in a less acidic environment**.** This is partly consistent with a previous study of Borges et al. (2011). According to their study, *L. monocytogenes* biofilms survived and even grew at the higher pHs investigated than the normal vaginal pH, suggesting that fetus from women having increased vaginal pH values during pregnancy may be at a higher risk of listeriosis.

Other studies (Skowron et al. 2019; Fan et al. 2020) showed that the biofilm formation of *L. monocytogenes* is significantly inhibited at pH 4 compared to neutral environment. Moreover, it was noticed that the production of some elements of the biofilm matrix (namely the extracellular polymeric substances) is dependent on the pH of the medium (Chaieb et al. 2007). The pH optimum of EPS secretion is around neutral pH (Tilahun et al. 2016), and EPS production has been shown to protect against environmental stressors such as pH (Bogino et al. 2013).

A



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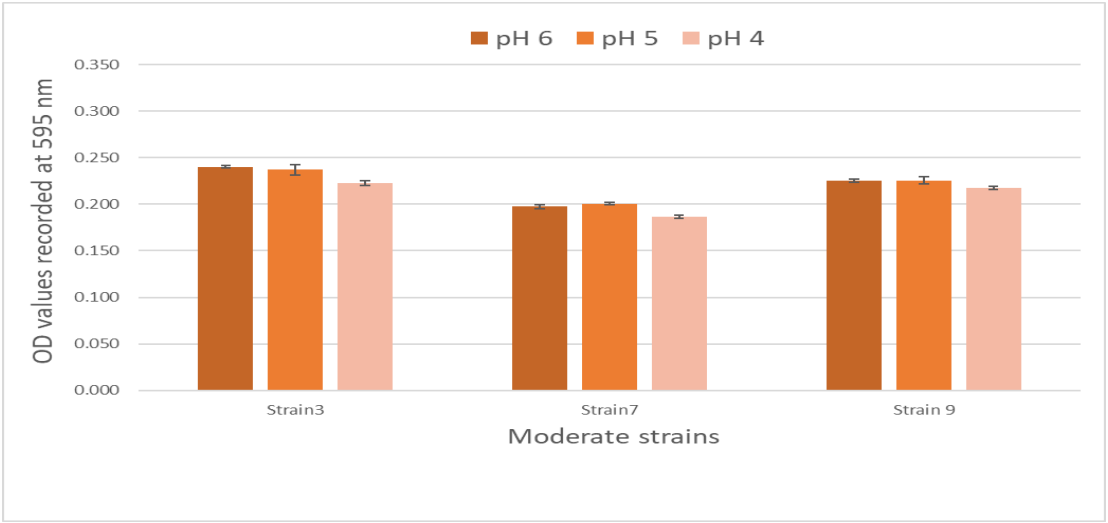

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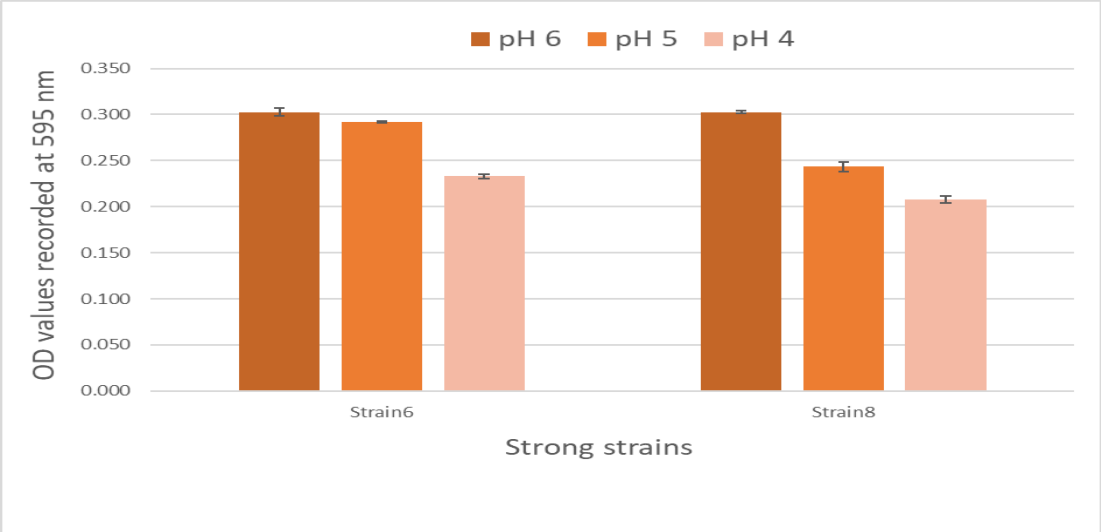
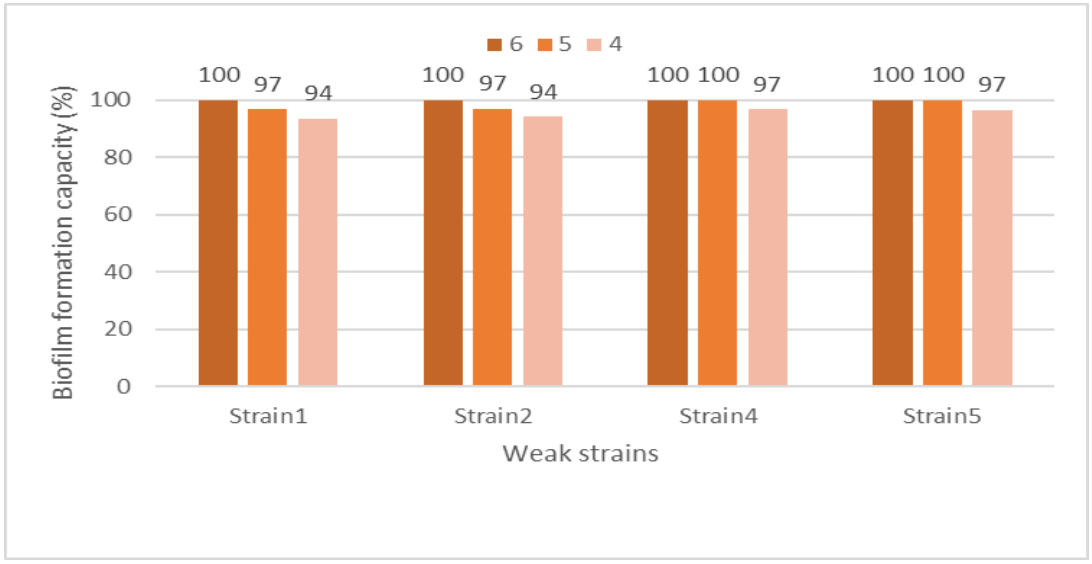


Figure 16. Biofilm formation of *L. monocytogenes* strains at pH 4, pH 5 and pH 6 (at 37 °C, when NaCl concentration was 0%) based on the measured optical density values at 595 nm. Error bars represent the standard deviations of the means, from three individual measurements.

A



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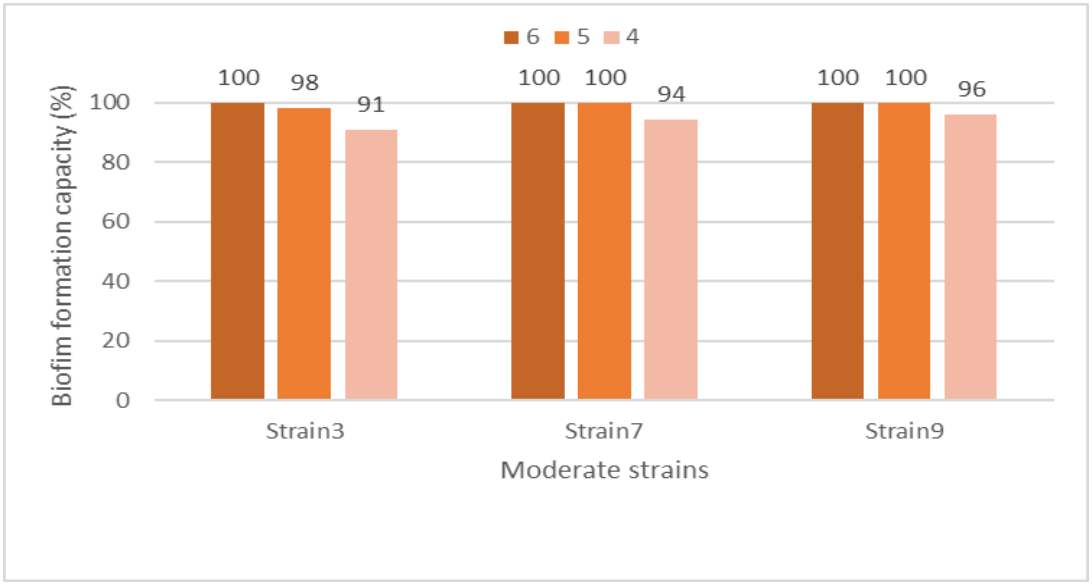

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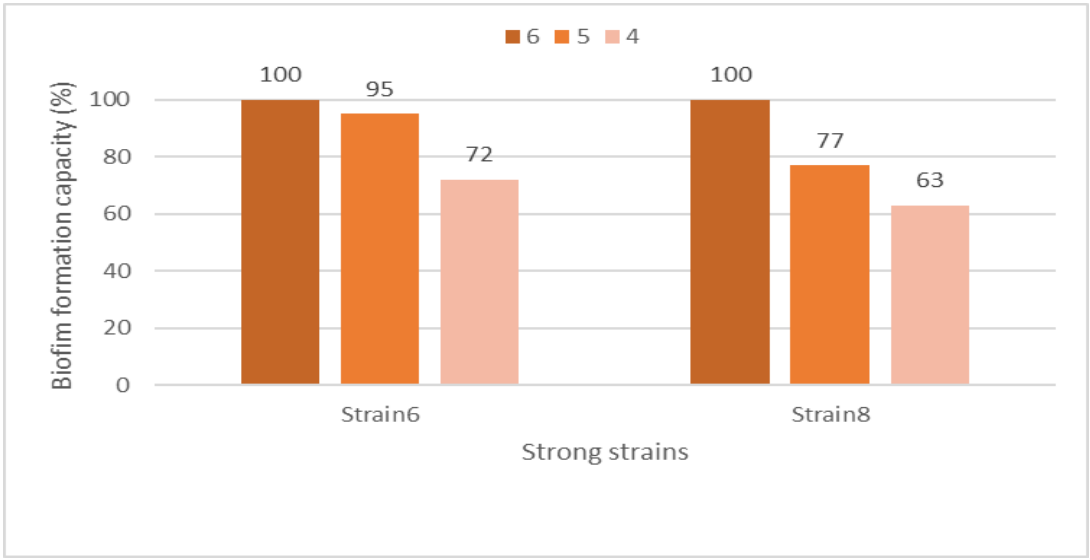


Figure 17. The effect of different pH values on biofilm formation capacity of *L. monocytogenes* strains incubated at 37 °C, 0% NaCl.

Concerning Group3, we found that the ability of the strain decreased with 32% compared to Group1 and Group 2 that showed 5 and 6 % decrease. We can conclude again that the strains which had originally stronger biofilm-forming capacity were the most sensitive to low pH.

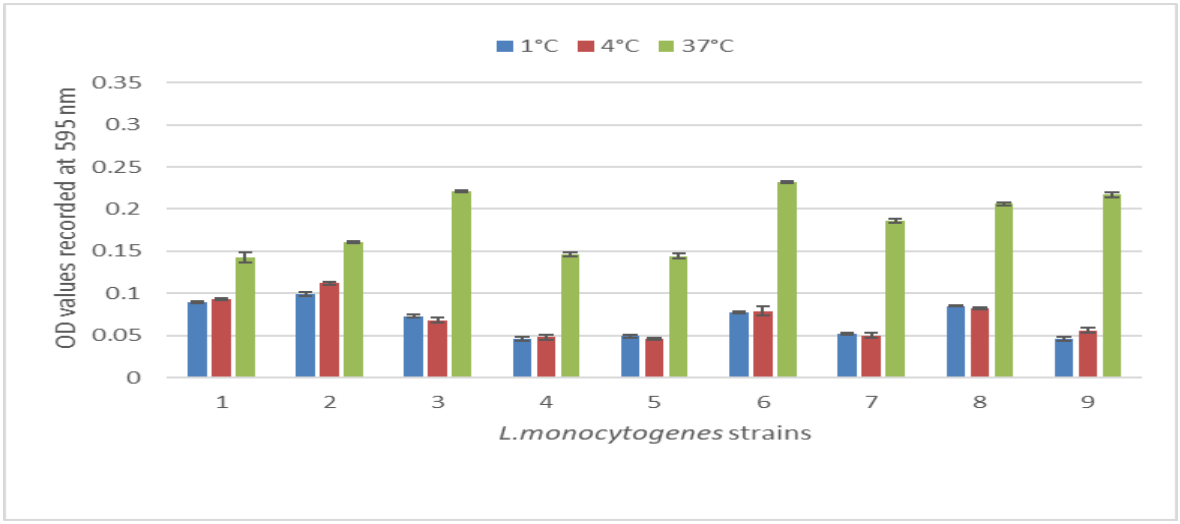
Among the examined *L. monocytogenes* strains, *L. monocytogenes* NCAIMB01966T (strain 6) and 5105 3a (strain 8) formed the highest amounts of biofilm (OD_{595} > 0.25), while the weakest biofilm-forming strain found to be *L. monocytogenes* CCM7202 (strain 5) showing an OD_{595}< 0.15 under the same conditions. Although, we can still conclude that most *L. monocytogenes* strains analyzed formed relatively good biofilm amounts at optimal conditions (37 °C, pH=6, NaCl 0%) in M9 Minimal Media.

The results of the individual treatments showed that the examined strains were different regarding to their biofilm-forming ability (low, moderate, and strong ability), therefore biofilm formation ability is strain dependent. The largest variability was found in the strongest biofilm-forming strains. This is consistence with the findings of Fan and co-workers (2020), they proved that in general, strong biofilm-forming strains are highly variable in response to environmental factors.

5.5 Effect of different stress conditions applied in combination on the biofilm formation of *L. monocytogenes* strains

The biofilm formation of different *Listeria monocytogenes* strains was analysed in M9 Minimal Media with 5% and 7.5%, NaCl concentration at different pH values and temperatures. The average of optical density recorded at 595 nm are shown in (Figure 18-19) and then the normalized capacity of the strains was calculated (Figure 20-21).

A



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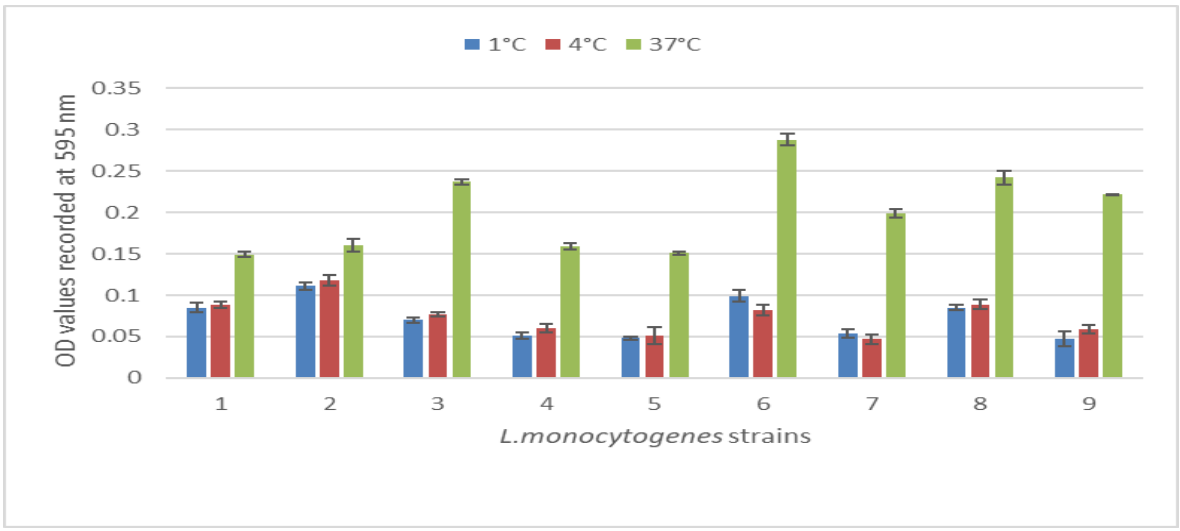

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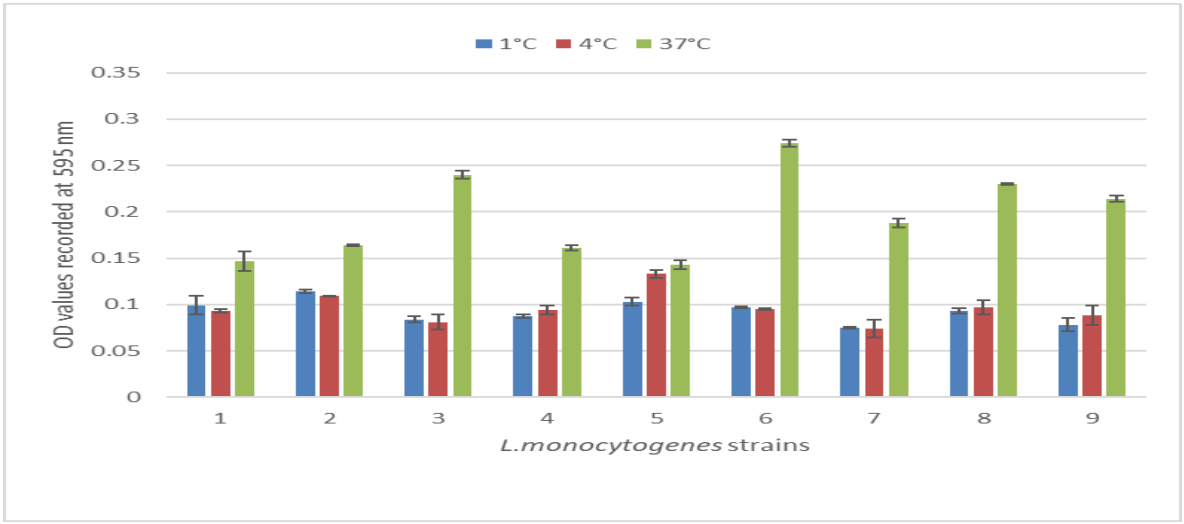
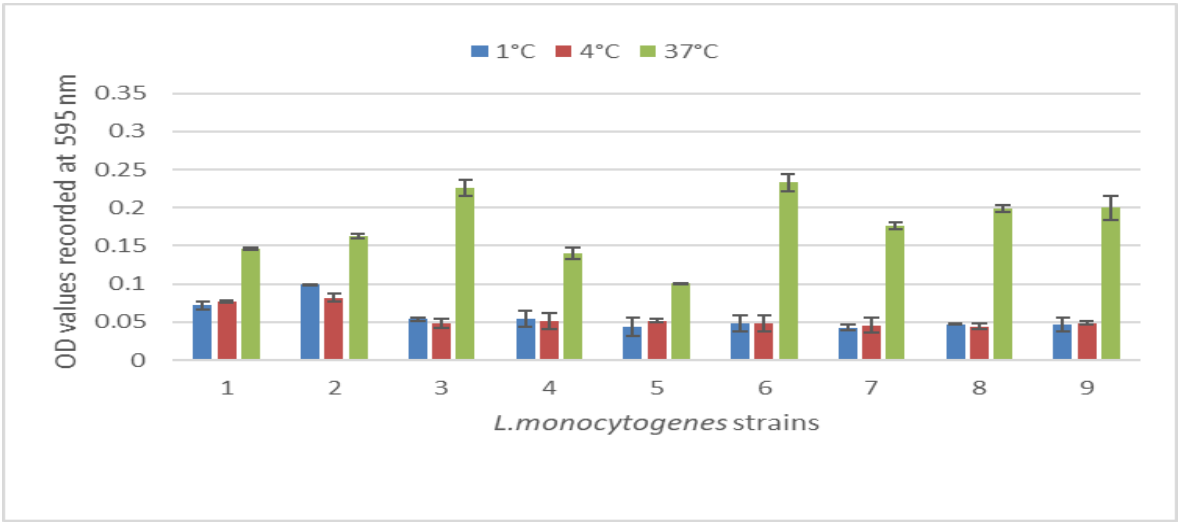
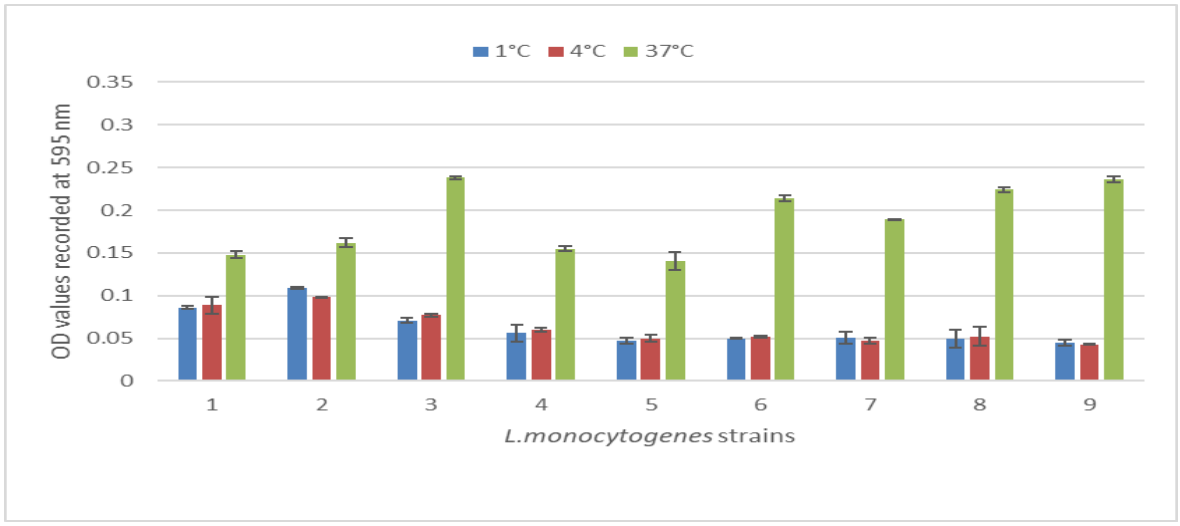


Figure 18. Biofilm formation of *L. monocytogenes* based on the measured optical density values at 595 nm under different pH 4 (A), 5 (B), and 6 (C) and temperatures (1, 4, and 37 °C) with 5% NaCl concentration. Error bars represent the standard deviations of the means, from three individual measurements.

A



B



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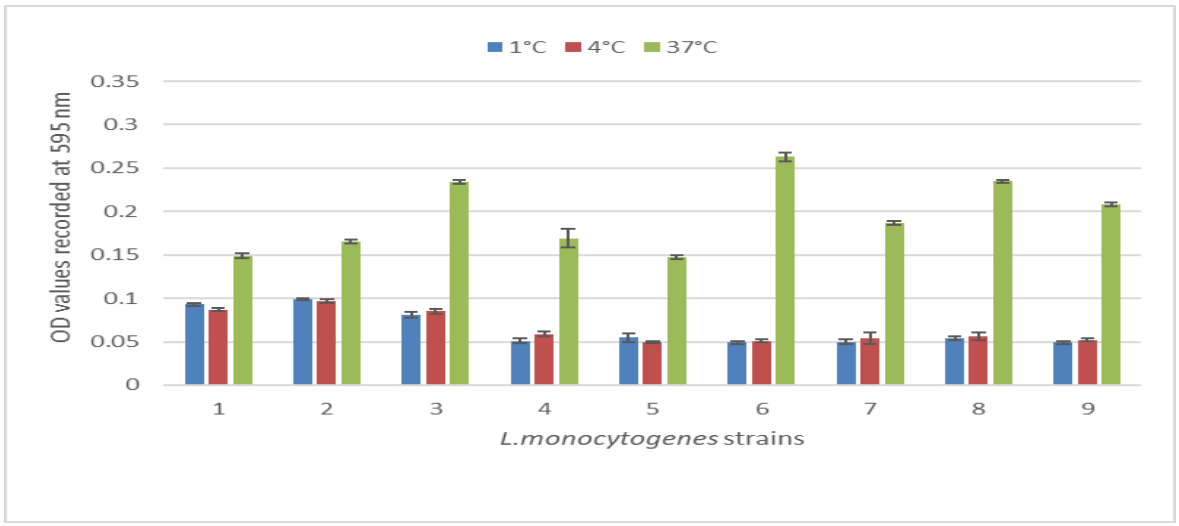


Figure 19. Biofilm formation of *L. monocytogenes* based on the measured optical density values at 595 nm under different pH values 4 (A), 5 (B), and 6 (C) and temperatures (1, 4, and 37°C) with 7,5% NaCl concentration. Error bars represent the standard deviations of the means, from three individual measurements.

Analysing the Figures 18-19, we could observe that temperature has the most dominant effect on the biofilm formation of *L. monocytogenes* when 5% NaCl were added to the media/broth. The tested pH values had not influenced the biofilm formation, we did not receive significant differences in the measured OD values. We received the same results when 7.5 % NaCl was added.

According to Figures 20-21, the biofilm formation capacity of *L. monocytogenes* in the presence of 5 and 7.5% NaCl at 37 °C was higher compared to the viable populations of *L. monocytogenes* when incubated at 1 °C and 4 °C. Regarding the pH, we didn't observe a significant difference between 4, 5, and 6. These results confirmed our findings from analysing the OD values.

The biofilm formation of different *Listeria monocytogenes* strains was analysed in M9 Minimal Media with 15% NaCl concentration at different pH values (4 and 6) and temperatures. The averages of optical density recorded at 595 nm are shown in Figure 22.

When 15 % NaCl was added, differences could be observed between the responses of the strains in terms of tested pH values at 37 °C. At pH=4 and at 37 °C OD≈0.1 value was measured for all strains.

These combinations were the best because in the individual treatments only 15% NaCl has a significant effect on the decline of the biofilm formation of *L. monocytogenes*, and there was no significant difference between 0 and 7.5 % NaCl (Figures 14 and 15). Similar tendency was observed for pH in the individual treatments. In fact, only pH 4 influenced the decrease of *L. monocytogenes* especially for strains 6 and 8.

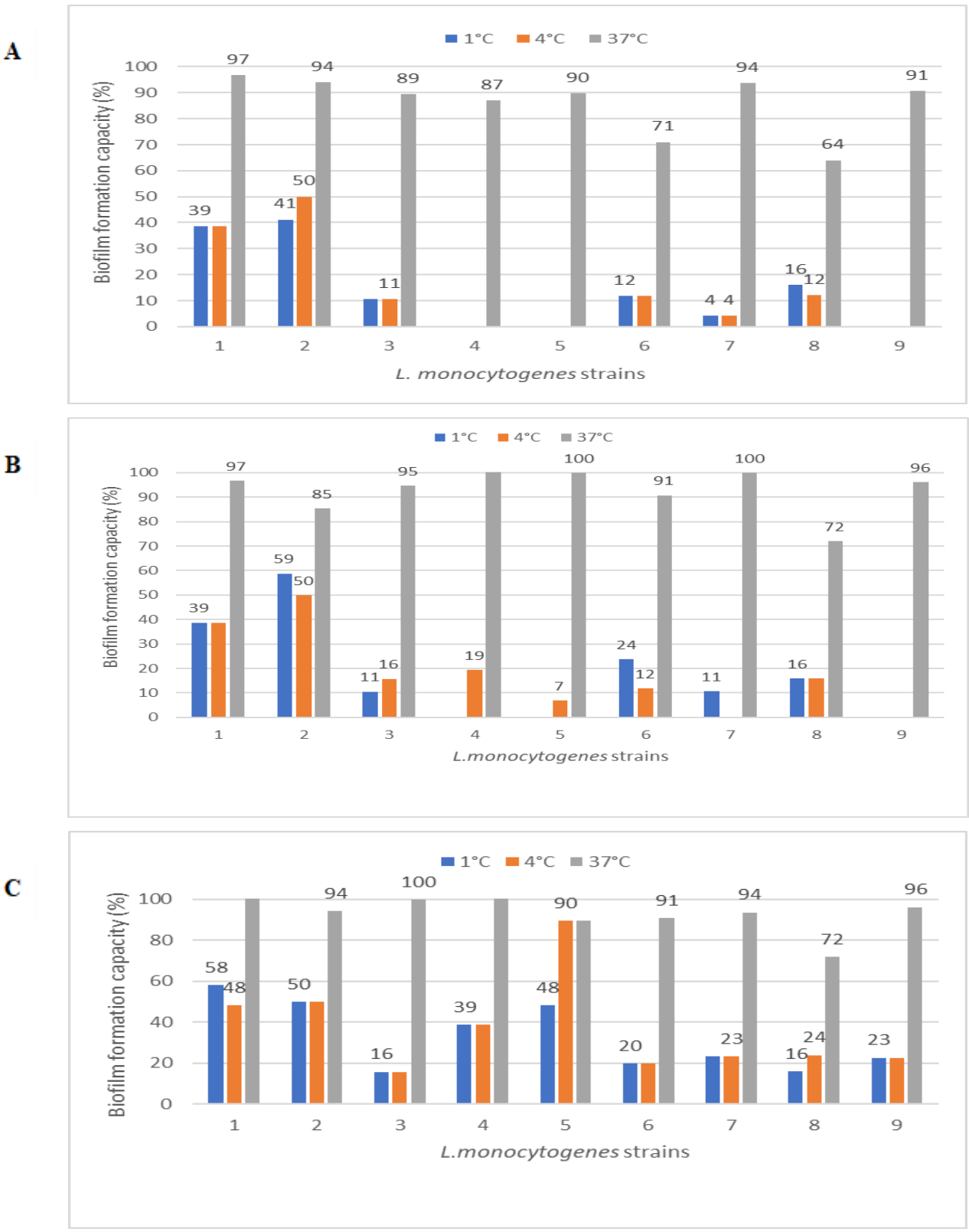


Figure 20. Biofilm formation capacity of *Listeria monocytogenes* under different pH 4 (A), 5 (B), and 6 (C) and temperatures (1, 4, and 37 °C) with 5% NaCl concentration.

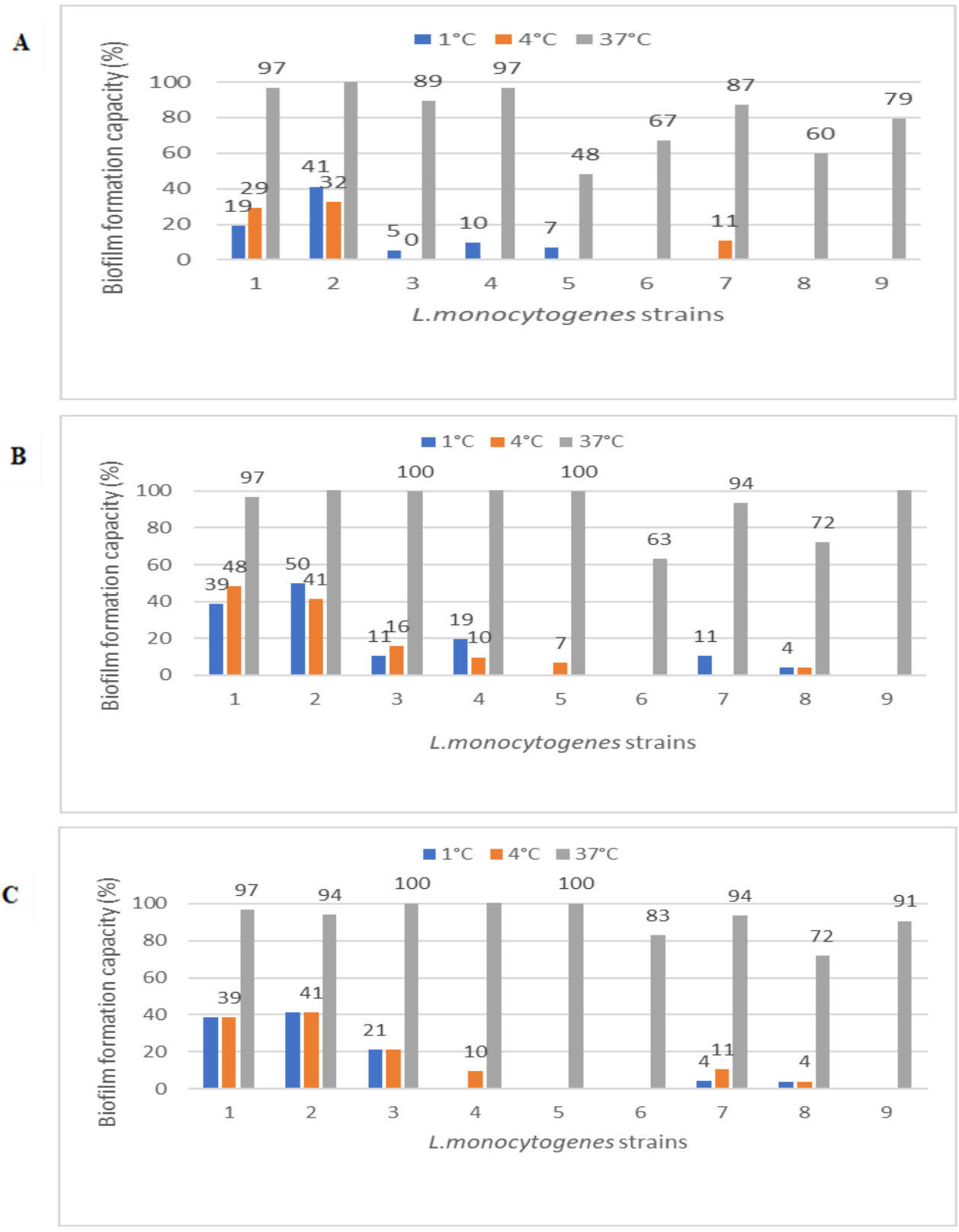
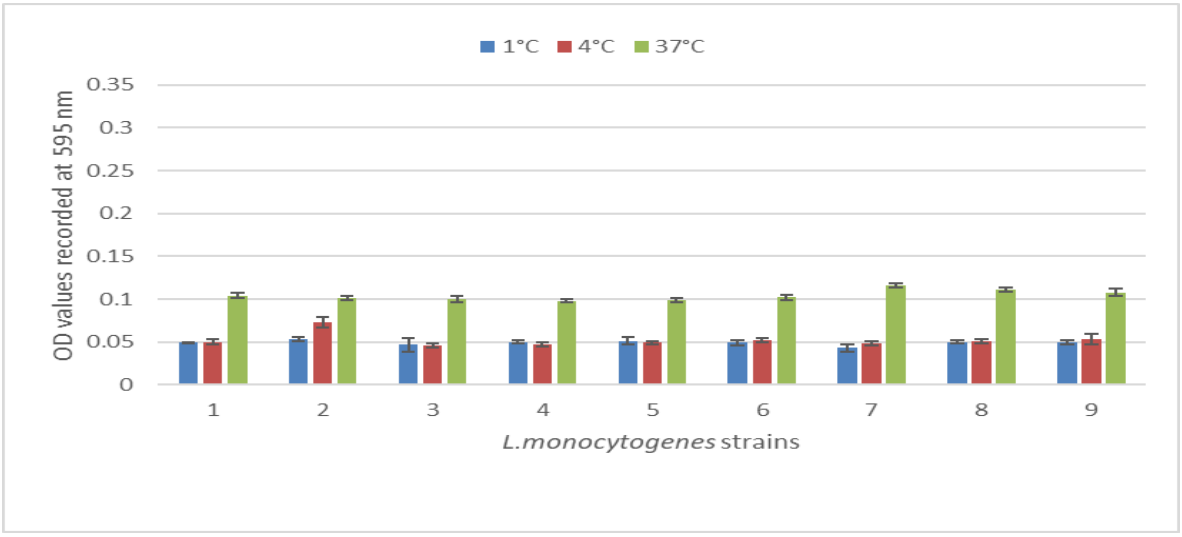


Figure 21. Biofilm formation capacity of *Listeria monocytogenes* under different pH 4 (A), 5 (B), and 6 (C) and temperatures (1, 4, and 37 °C) with 7.5% NaCl concentration.

A



B

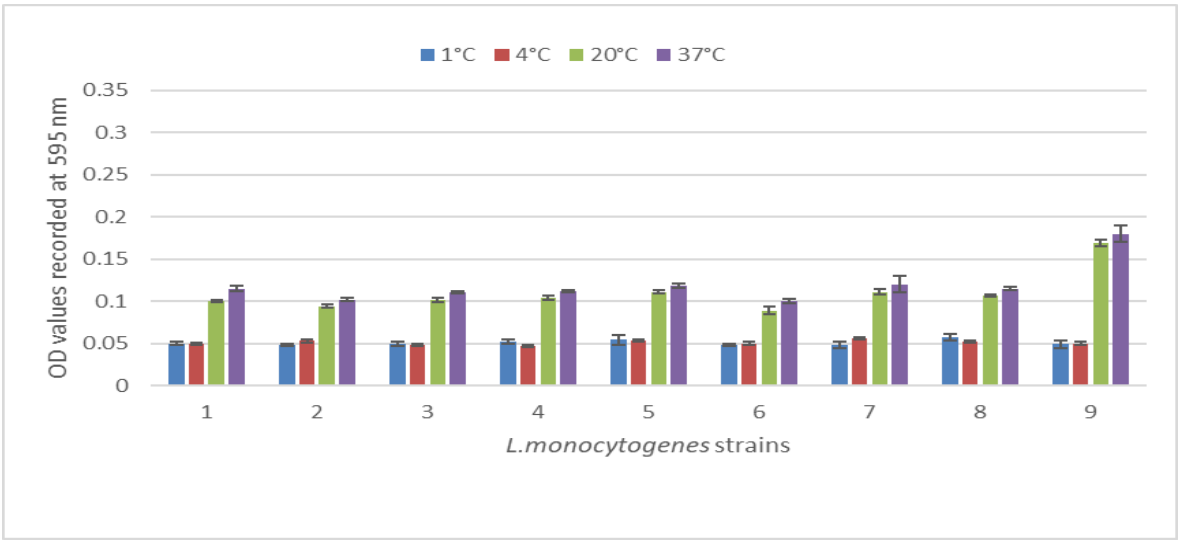


Figure 22. Biofilm formation of *L. monocytogenes* based on the measured optical density values at 595 nm under different pH values 4 (A) and 6 (B), and temperatures (1, 4, 20, and 37°C) with 15% NaCl concentration. Error bars represent the standard deviations of the means, from three individual measurements.

According to Figure 23, the biofilm formation capacity of *L. monocytogenes* in the presence of 15% NaCl declined strongly within 2 days of incubation at 37 °C compared to the viable populations of *L. monocytogenes* when incubated at the same temperature but with 5% or 7,5% of NaCl (Figure 20 and 21). So, we can conclude that it was more efficient to use 15% NaCl applied in combination with low temperatures. Temperatures at 4 °C and 1 °C had the most relevant effect in terms of inactivating the biofilm formation of *Listeria monocytogenes* strains.

These results indicate that the viability of *L. monocytogenes* strains is essentially influenced by refrigeration as well as by the additional stress effect of NaCl. However, these two parameters (15% NaCl and low temperature) inhibited the rates of growth of *L. monocytogenes* strains but were not lethal.

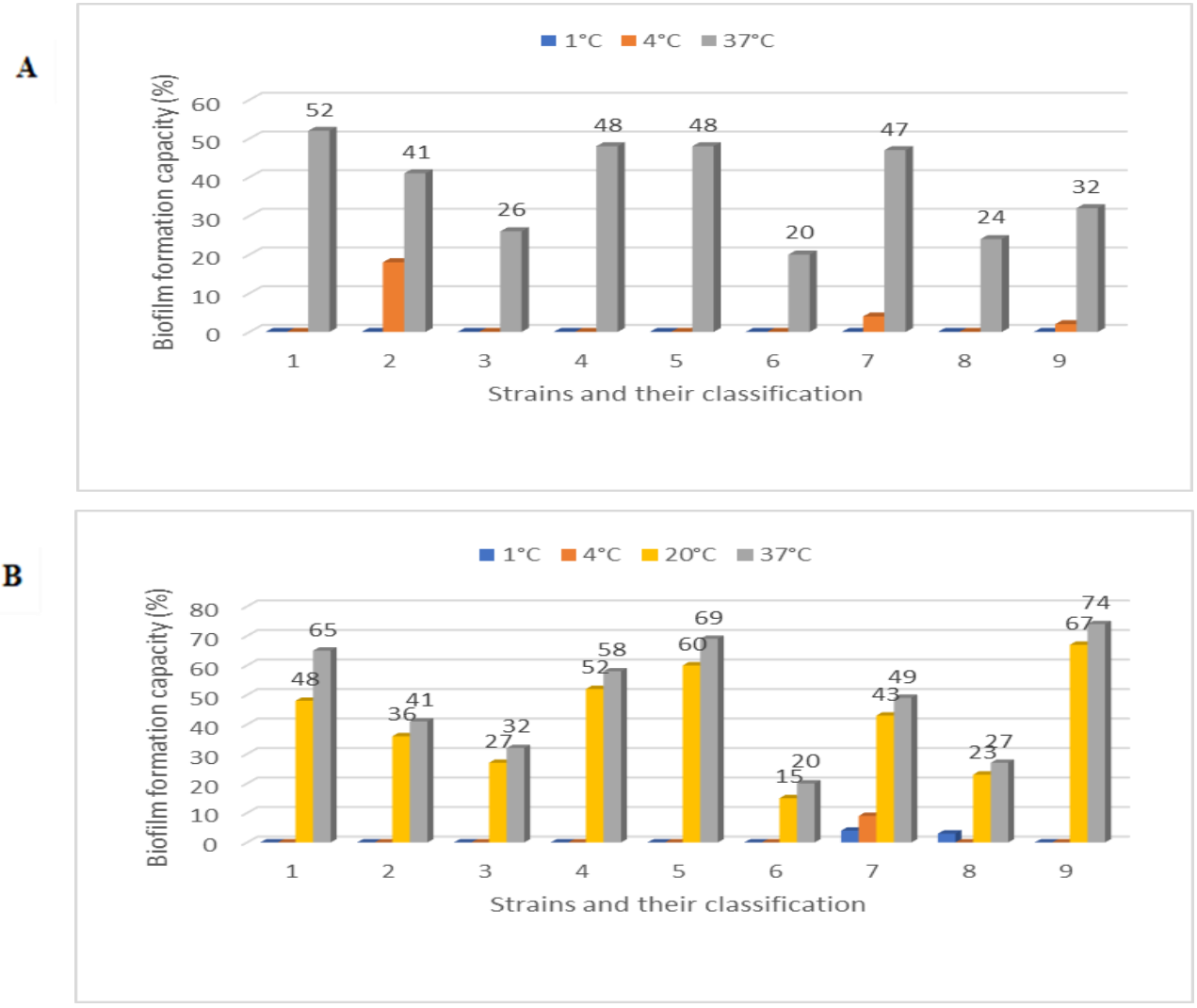


Figure 23. Biofilm formation capacity of *Listeria monocytogenes* under 15% NaCl concentration, pH 4 (A) and 6 (B), and different temperatures (1, 4, 20 and 37°C).

Concerning the pH, strain 9 (CCM 4699) was resistant at 37 °C when pH=6 and NaCl concentration 15%, but with the combination of pH4 (acidic environment), it was inhibited. While, at 4 °C the same pH enhanced the biofilm formation of the strain 2 (3b T1) showing adaptation to pH4.

We found that *L. monocytogenes* can grow at refrigeration temperatures (1 and 4 °C) in a wide pH range, with a high concentration of salt (15% NaCl). This is consistent with the findings of Cole et al., 1990. According to them, at 4 °C *L. monocytogenes* can survive and grow in culture media with up to 10 and 12% NaCl.

Our results showed that the combined treatments had negative effect on the biofilm formation of *L. monocytogenes* compared to the results of individual stresses. We also found that the strong biofilm formers were more sensitive to all the treatments compared to the moderate or weak biofilm forming groups, since their biofilm forming capacity (%) decreased more compared to the two other groups (moderate or weak). This may suggest that since the originally weak or moderate biofilm formers were more resistant to the treatments, they can bring survival advantages to the strains. The results are also important from a practical point of view, as the conditions employed in these experiments correspond reasonably well to those that occur during the process of cheese production (i.e. a decrease in pH values during the ripening stage, and an increase in NaCl content).

Different amounts of biofilms were generated by the investigated *Listeria monocytogenes* strains as an answer to different stresses, and their biofilm formation was influenced by a multitude of environmental parameters. In consequence, each strain has its own way and its own response when submitted to stress conditions like high concentration of sodium chloride, different temperatures, or low pH values. The differences in tolerance among these strains highlight the possibility for the adapted strains to alter biofilm formation in response to changing environmental factors. This may result in the formation of persistent strains that can survive and even grow in varying environments over long periods of time in food processing environment.

5.6 Effect of sub-lethal temperature on the biofilm formation of *L. monocytogenes* strains

The biofilm formation of *Listeria monocytogenes* strains was analyzed in M9 Minimal Media following heat treatment (50 °C), the results of 3 repetitions can be seen in Figure 24.

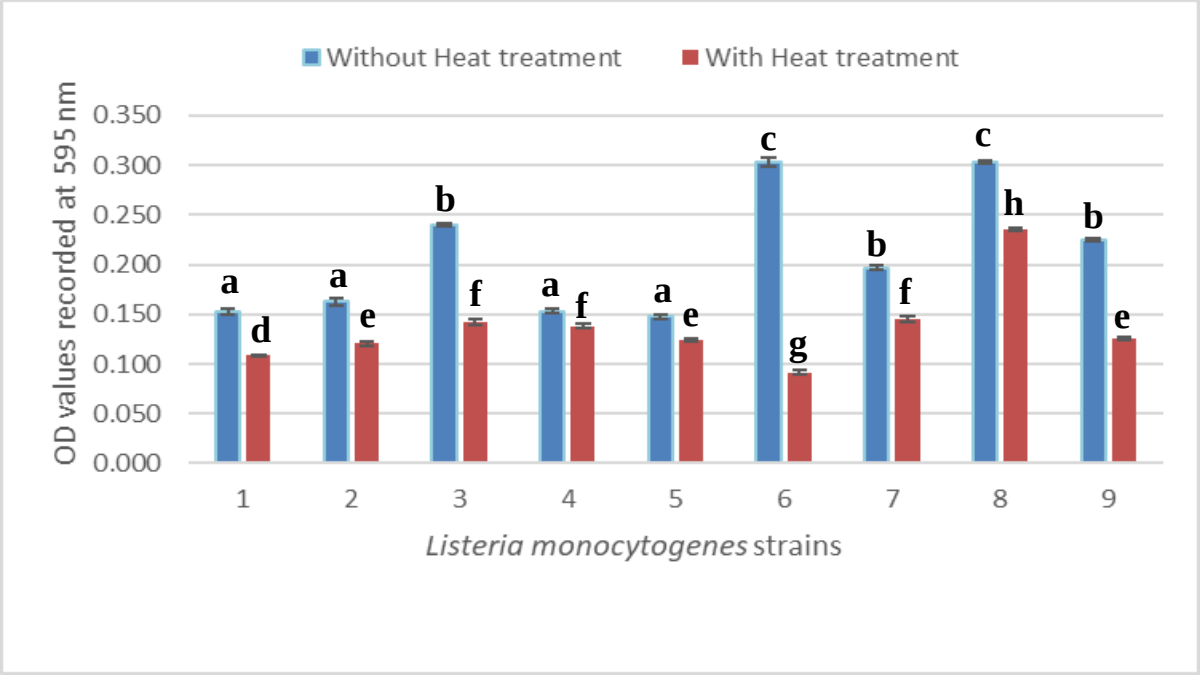


Figure 24. Biofilm formation of *L. monocytogenes* strains with and without heat treatment. Error bars represent the standard deviations of the means, from three individual measurements. Different letters above the bars indicate a significant difference (P≤0.05).

All *L. monocytogenes* strains formed biofilm in a lesser extent when they were treated by high temperature (50 °C). This is consistent with the findings of Lado and Yousef (2007). They proved that temperatures above 50 °C are lethal to *L. monocytogenes*.

L. monocytogenes 5105 3a (strain 8) as a strong biofilm forming strain showed resistance to high temperature. Although strains CCM4699 (strain 9) and CCM5576 (strain 4) proved to be weak and moderate biofilm formers compared to other strains they also showed considerable resistance compared to their non-heat-treated counterparts. According to similar studies, different strains prefer different temperatures and media when forming biofilms (Pan et al. 2010).

We found that the heat treatment enhances the inhibition of *L. monocytogenes*, and it decreased their associated biofilm formations. However, some strong and even weak biofilm forming strains showed considerable resistance to intermediate heat.

5.7 Effect of antimicrobials on *L. monocytogenes* biofilms

The effect of chlorine, acetic acid, balsamic vinegar (undiluted), white vinegar, and lactic acid against the growth of NCAIM B1966 (strain 6) and CCM 5576 (strain 4) after 24h and 48h of incubation was examined. These two strains were selected based on the results of previous experiments as a strong biofilm former and as a weak biofilm former. The results of 3 repetitions are shown in Figures 25 and 26 respectively.

L. monocytogenes reductions obtained after washing treatments were relative to populations on inoculated lettuce. The inoculation level used in this experiment was higher than natural contamination to allow valid results of bacterial reductions after washing with different solutions.

This experiment confirmed that the usual method of dipping lettuce with water used in home and in retail environments is not effective in removing *Listeria* from lettuce since the viable *L. monocytogenes* found is relatively high.

Examining Figure 25, all the treatments used in this experiment decreased the viable count of *L. monocytogenes* after 24 hours of incubation compared to the water treatment, except the treatment with the lactic acid. In case of this treatment, we observed just a slight decrease in the viable count of *L. monocytogenes* (almost the same reduction found after washing with water). Concerning the white vinegar and the acetic acid solutions, these 2 treatments showed similar efficiency on removing *L. monocytogenes* from lettuce.

The two best treatments with the highest bacterial reductions were chlorine solution and balsamic vinegar ($P \leq 0.05$). The efficacity of chlorine was expected since it is widely used in the food industry to kill bacteria and sanitize the food processing environment (e.g. washing fruit and vegetables and sanitizing food contact surfaces), in addition in a previous study of Aarnisalo and co-workers (2000) a chlorinated alkaline cleaner was found to have good disinfecting properties against *L. monocytogenes*.

The efficacity of balsamic vinegar to decontaminate *L. monocytogenes* from lettuce surfaces was slightly lower than chlorine. The high antimicrobial activity of balsamic vinegar solutions was not only due to hydrogen ion effect but may also related to the presence of compounds with antimicrobial properties resulting from the fermentation of grape juice. It is known that grapes

contain several phenolic compounds that exhibit antilisterial activity, particularly polymeric phenolic compounds (Baydar et al. 2004, Oliveira et al. 2013).

After 48h of incubation, (Figure 26) the population of *L. monocytogenes* slightly increased compared to the viable *L. monocytogenes* found after 24h, and again, the white vinegar and acetic acid treatments showed almost similar efficiency in removing *L. monocytogenes* from lettuce.

After 48h of incubation, balsamic vinegar resulted in the greatest reductions in the viable number of *L. monocytogenes,* showing a similar or higher reduction compared to chlorine. The presence of phenolic compounds naturally present in grapes may be responsible for their high antibacterial activity. Also, we observed that the colour and texture of the lettuce were retained when treated with balsamic vinegar while the other treatments showed significant colour change and softening.

The decrease in the efficacity of sanitizing treatments may be explained by the initiation of biofilm formation whereas there is an increase in the development of cell aggregates after 24 h of incubation (Ells and Hansen, 2006). The strength of attachment is the main factor affecting the efficacy of sanitizing treatments (Ölmez and Temur, 2010).

To summarize, all tested solutions showed higher bactericidal effects against the *L. monocytogenes* strains (6 and 4) than water, while the balsamic vinegar activity was clearly higher especially, after 48h. Balsamic vinegar showed similar and even better effectiveness than chlorine in inhibiting *L. monocytogenes* on lettuce surface, and this seems to be a promising method to reduce *L. monocytogenes* presence in fresh produce at home and retail environments.

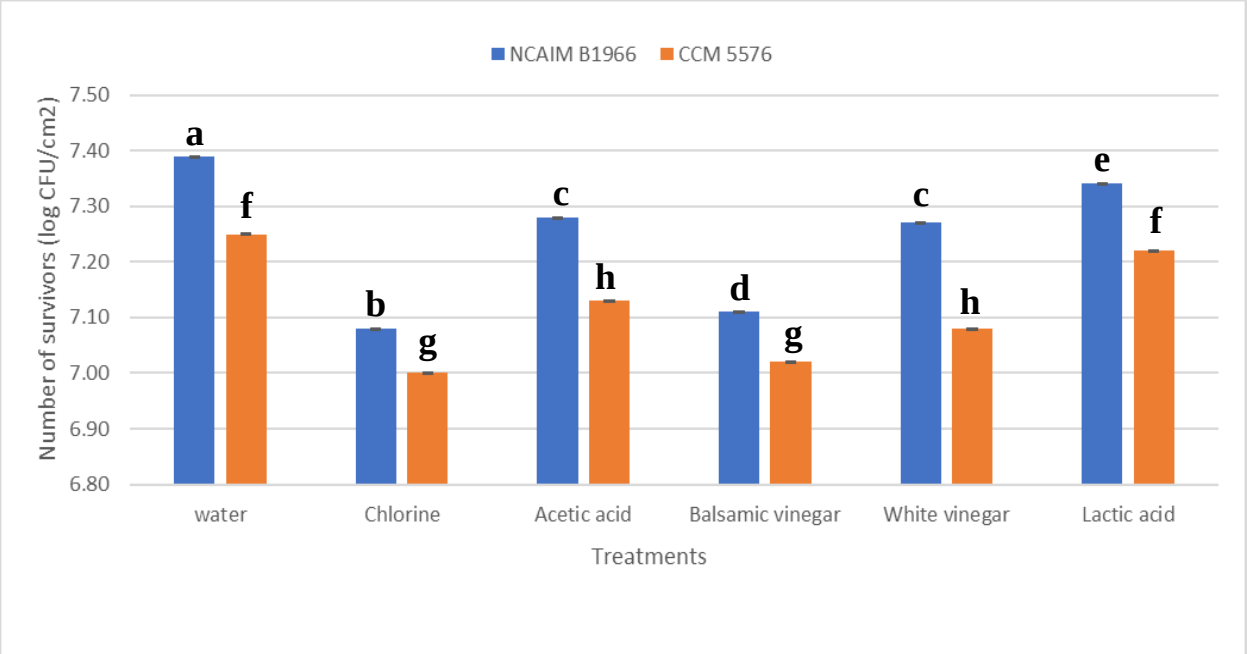


Figure 25. Antilisterial activities of different washing solutions after 24h of incubation. Error bars represent the standard deviations of the means, from three individual measurements. Different letters above the bars indicate a significant difference (P≤0.05).

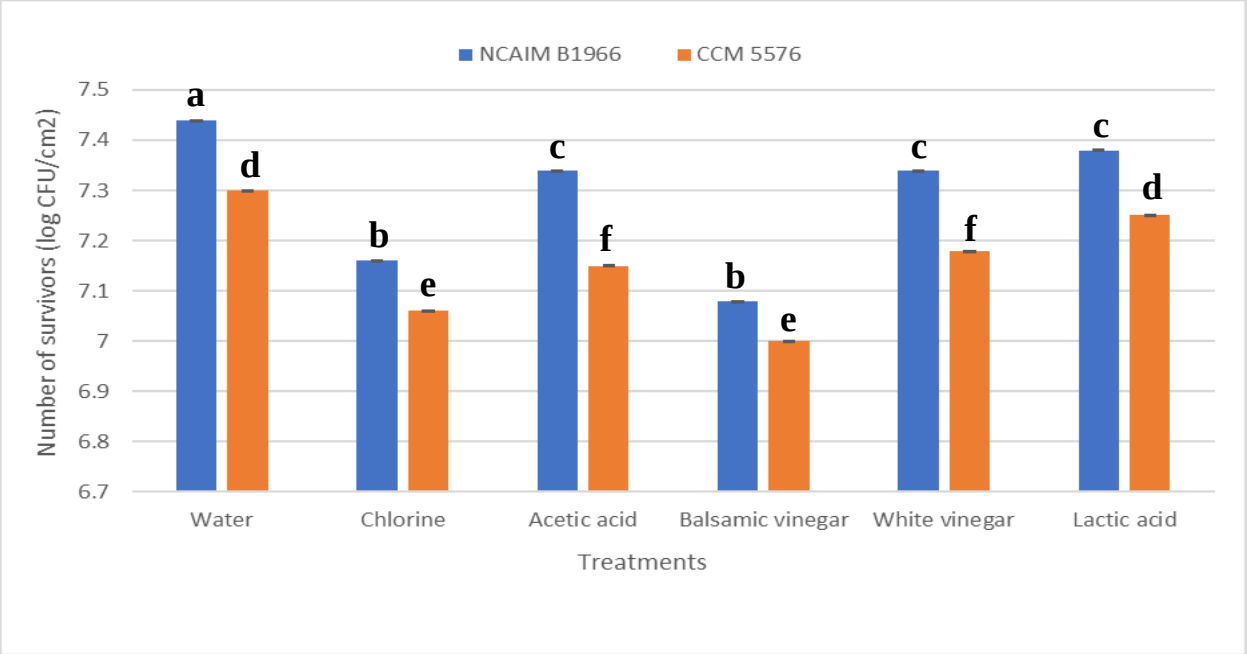


Figure 26. Antilisterial activities of different washing solutions after 48h of incubation. Error bars represent the standard deviations of the means, from three individual measurements. Different letters above the bars indicate a significant difference (P≤0.05).

6. CONCLUSIONS AND RECOMMENDATIONS

The results reported here demonstrate that strong biofilm former of *Listeria monocytogenes* strains produced more biofilms under the investigated environmental conditions, indicating strain-dependent biofilm formation. *L. monocytogenes* is tolerant to environmental stresses (applied individually or in combination) such as high NaCl concentrations, refrigeration temperatures, and low-pH conditions. The different treatments had a more expressed effect in the case of strong biofilm former strains, but we can conclude that the temperature treatment was the most active inhibitor followed by the NaCl and the pH.

The sub-lethal heat treatment accentuates the inhibition of *L. monocytogenes*, and it decreased their associated biofilms. However, some strong and even weak biofilm forming strains showed considerable resistance to intermediate heat. Although, a detailed study is needed to better identify other factors affecting the growth and death of *Listeria* in foods.

All tested solutions (chlorine, acetic acid, balsamic vinegar, white vinegar, and lactic acid) showed higher bactericidal effects against the *L. monocytogenes* strains than balsamic vinegar having a clearly higher activity. Balsamic vinegar may be a promising effective solution to inhibit other food pathogens present on produce surface or other foods. There is a lack of studies with these vinegars, and it is an important resource for households and food establishments due to is availability and organic nature.

In the future, it is proposed to use new analytical methods to study the structure and formation of *Listeria monocytogenes* and other food borne pathogenic bacterial biofilms in vivo and in vitro, e.g. laser scanning microscopy etc.

As the use of enzymes is becoming increasingly important in the fight against biofilms, it would be useful to investigate their effect (e.g. the application of pectinase, arabanase, cellulase, hemicellulase, beta-glucanase, and xylanase activities on *Listeria monocytogenes* biofilms). In addition to enzymes, photoinactivation is also a possible method for inactivating biofilms. This would represent an additional potential for inactivation of *Listeria* species and other foodborne pathogens, either on food contact or on food surfaces such as leafy vegetables e.g. lettuce.

7. NEW SCIENTIFIC RESULTS

1) Based on the optical density readings in optimal conditions, in term of the biofilm-forming ability, the examined 9 strains of *L. monocytogenes* could be divided into 3 groups: weak (NCAIM B1454, 3b T1, CCM5576, CCM7202), moderate (11/4.12t03, 10887 ½ a, CCM 4699), and strong (NCAIM B01966T, 5105 3a) biofilm formers. Based on their normalized OD values (Labidi et al., 2023):

- it was found that strains which had originally strong biofilm-forming capacity were more sensitive to the temperature effect (their reduction in biofilm forming capacity with decreasing temperature was significantly higher compared to weak biofilm formers (<p 0.05)).
- it was shown that strains which had originally higher biofilm-forming capacity were more sensitive to the sodium chloride treatment (their reduction in biofilm forming capacity with increasing NaCl concentration from 5% to 15% was significantly higher compared to weak biofilm formers (<p 0.05)).
- it was proved that strains which had originally stronger biofilm-forming capacity were the most sensitive to low pH (pH 4) (their reduction in biofilm forming capacity with decreasing pH from 6 to 4 was significantly higher compared to weak biofilm formers (<p 0.05)).
- The results evidenced that originally weak or moderate biofilm formers were more resistant to the effects of temperature, NaCl and pH which can bring survival advantages to these strains.

2) Undiluted balsamic vinegar proved to be as effective as chlorine in inhibiting *L. monocytogenes* on lettuce surface which provide a promising method to reduce *L. monocytogenes* presence in fresh produce at home and retail environments.

3) It was presented for the first time that *Listeria monocytogenes* is able to form biofilms at the lower limit of its growth temperature range (1 °C). This property seems to be strain independent as all ten strains tested showed it.

8. SUMMARY

Food-borne diseases present a major problem throughout the world causing thousands of deaths. *L. monocytogenes* has been isolated from a wide range of foods and all of them have been associated with major outbreaks.

Biofilm formation by *Listeria* species is generally associated with their persistence in the food-processing environment. In this *Listeria* biofilm formation study, we have gained some idea about how different stress conditions such as salt concentration, temperatures, and pH affect the biofilm formation of different *Listeria* strains. Using a m assay with crystal violet staining, the biofilm formation of 15 different *Listeria* strains in M9 Minimal Media with varying concentrations of sodium chloride (from 0% to 15%), at different pH values (4, 5 and 6), and at different temperatures (1, 4, 20, and 37 °C) were examined separately and in combination.

The effect of sub-lethal temperature (50 °C) on the biofilm formation of *L. monocytogenes* strains was also examined. Additionally, the effect of some disinfectant (chlorine, acetic acid, balsamic vinegar, white vinegar, and lactic acid) on the growth of two strains NCAIM B1966 (strain 6) as a strong biofilm former and CCM 5576 (strain 4) as a weak biofilm former was also studied on lettuce leaves.

Examining our strains in optimal conditions, we found that the 9 strains of *L. monocytogenes* could be divided into 3 groups: weak, moderate, and strong biofilm formers.

In general *L. monocytogenes* strains showed higher biofilm forming ability than other examined *Listeria* strains. All *L. monocytogenes* showed decreasing patterns of biofilm formation when the sodium chloride concentration increased from 0 to 15%, especially for the strong biofilm formers they were the most sensitive to the sodium chloride treatment. Same results obtained for the non-pathogenic *Listeria* strains, which higher concentrations of sodium chloride did not present a stress condition that enhance the biofilm forming ability.

Regarding the temperature effect, as it was expected only 1 and 4 °C had the most relevant effect in terms of inactivating the biofilm formation of *Listeria monocytogenes* strains. Also, we found that the strains which had originally lower biofilm-forming capacity were less sensitive to the temperature effect.

The decrease of pH showed an inhibition effect for biofilm formation of all the non-pathogenic *Listeria* strains which formed lower amounts of biofilms than most of pathogenic *L. monocytogenes* strains. However, we found that *L. monocytogenes* prefer to grow and form biofilms in an acidic environment especially for strains 6 and 8.

The combination of these treatments (15% NaCl concentration and low temperatures) resulted better inhibition of biofilm formation of *L. monocytogenes* compared to the results of individual stresses. We also found that the strong biofilm formers were more sensitive to all the treatments compared to the moderate or weak biofilm forming groups.

L. monocytogenes is tolerant to environmental stresses (applied individually or in combination). But we can conclude that the temperature treatment was the most active inhibitor followed by the NaCl and the pH.

Studying the effect of sub-lethal temperature on the biofilm formation of *L. monocytogenes* strains. We found that the heat treatment accentuates the inhibition of *L. monocytogenes*, and it decreased their associated biofilms. However, some strong and even weak biofilm-forming strains showed considerable resistance to intermediate heat.

Examining the effect of some disinfectants on the growth of the selected two strains of *L. monocytogenes*, we found that the lactic acid is not efficient in removing *L. monocytogenes* from lettuce, the white vinegar, and the acetic acid solutions showed similar efficiency and they were better than the lactic acid.

The two best treatments with the highest bacterial reductions were chlorine solution and balsamic vinegar. The efficacity of balsamic vinegar to decontaminate *L. monocytogenes* from lettuce surfaces was similar or higher to chlorine, and this seems to be a promising method to reduce *L. monocytogenes* present in product at home and retail environments since it is a natural product.

Further investigation on the biofilm formation of *Listeria* strains and their interaction under different environmental conditions are still necessary to provide more information on conditions that may inhibit biofilm formation and could be used to control the production of biofilms in food industry. Further investigations are needed also to study the effect of vinegars, since they are an important resource for households and food establishments due to its availability and organic nature.

9. APPENDICES

A1. BIBLIOGRAPHY

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A.2. STATISTICAL ANALYSIS

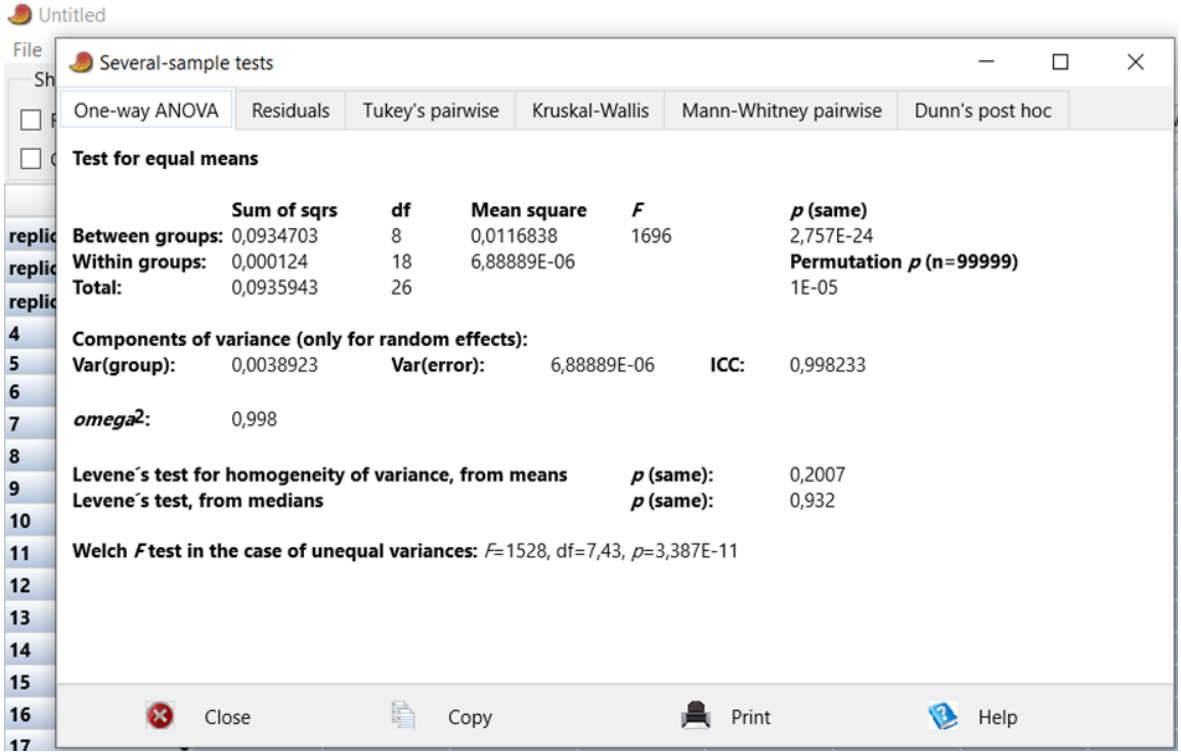

Figure 1. Past program one way ANOVA_Without heat treatment

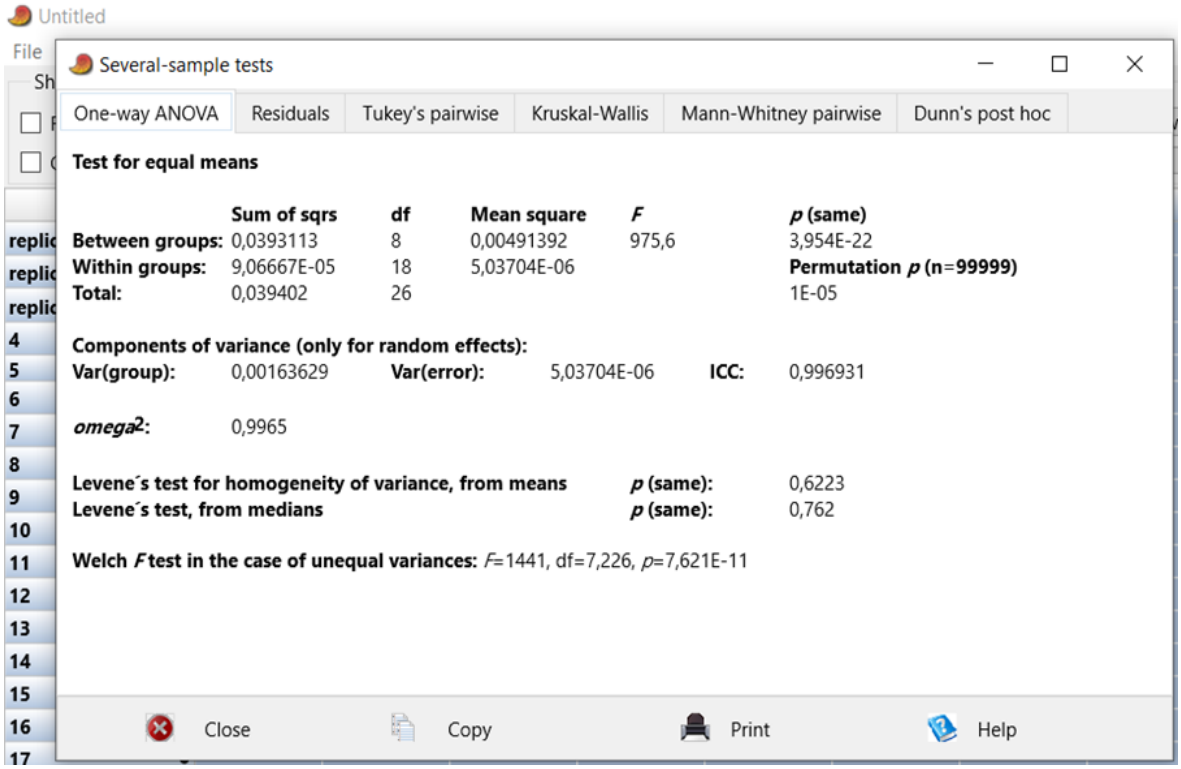

Figure 2. Past program one way ANOVA_ With heat treatment

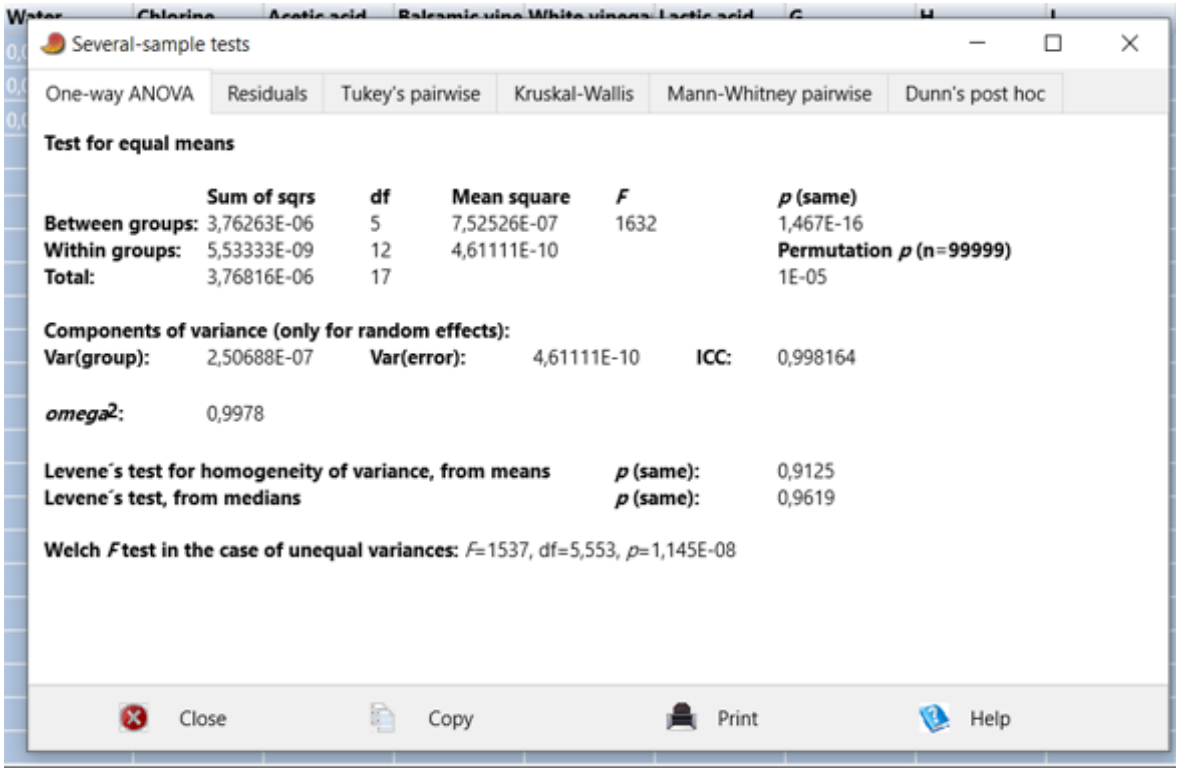

Figure 3. Past program one way ANOVA for NCAIM B1966 after 24h

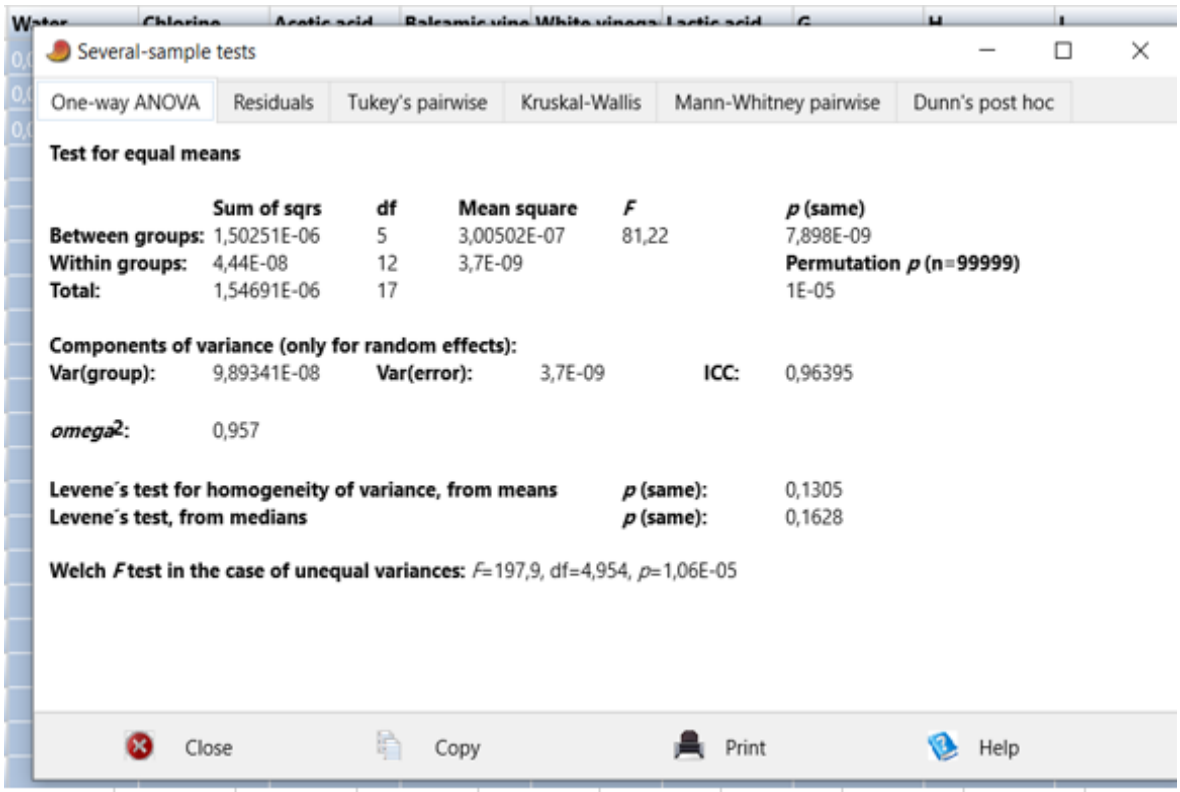

Figure 4. Past program one way ANOVA for CCM 5576 after 24h

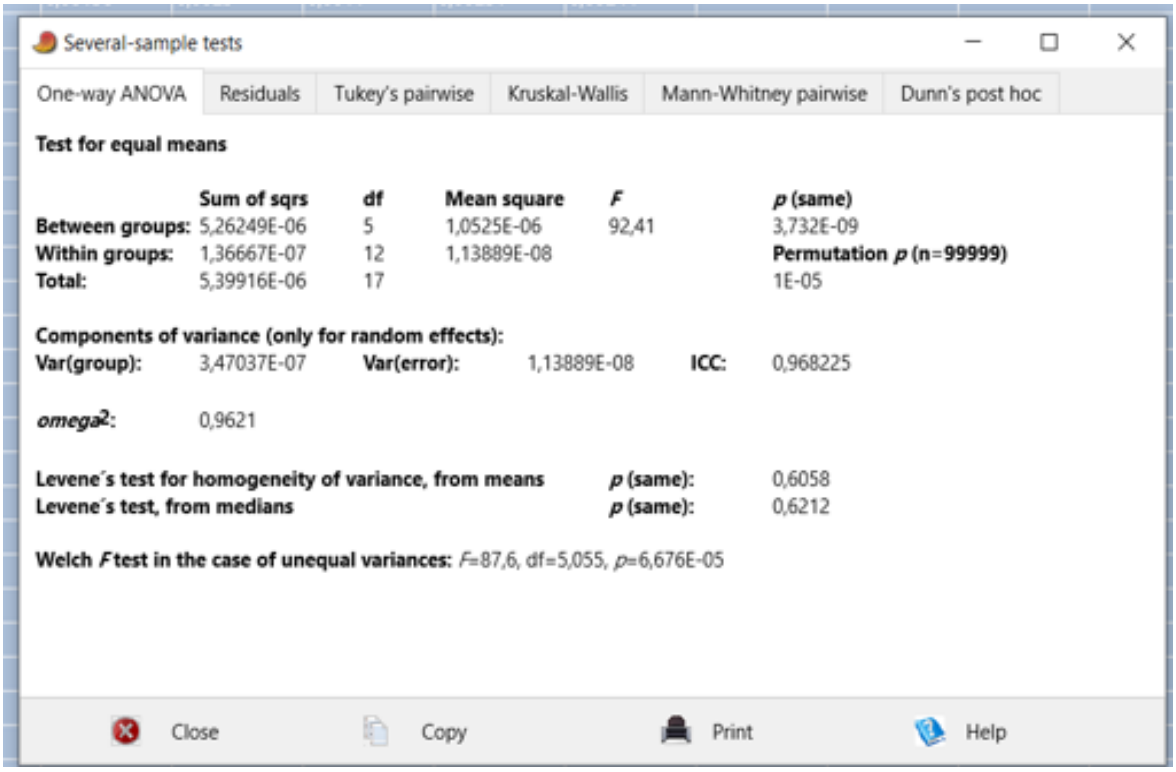

Figure 5. Past program one way ANOVA for NCAIM B1966 after 48h

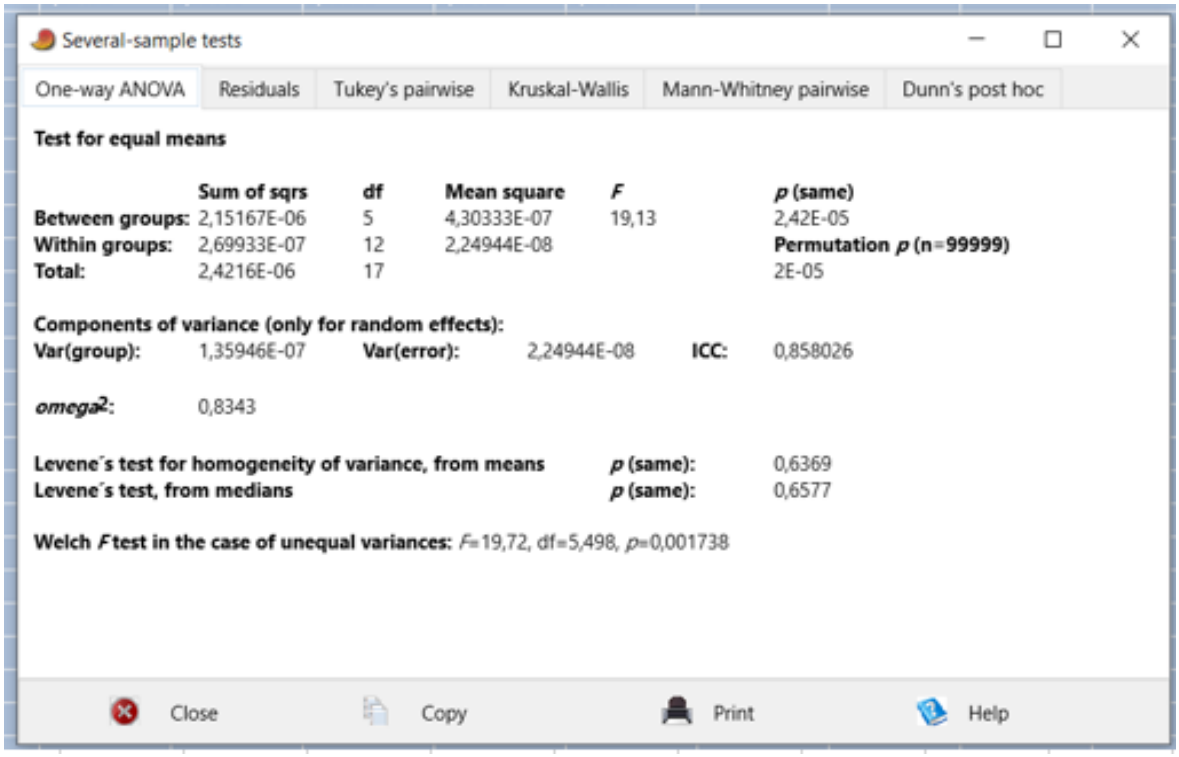

Figure 6. Past program one way ANOVA for CCM 5576 after 48h

A3. LIST OF FIGURES

A4. LIST OF TABLES

10. ACKNOWLEDGMENTS

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