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The effect of wine on the survival and invasiveness ability of *Listeria monocytogenes*

THESIS

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To my mother, father and brother we might be scattered around the world yet so close

Resumo

Listeria monocytogenes é um patogénio de origem alimentar conhecido pela gravidade da sua infeção, a listeriose, e pela sua capacidade de crescer em condições adversas, como temperaturas de refrigeração, ampla gama de valores de pH, atividade de água baixa e elevada salinidade. *L. monocytogenes* pode induzir a sua própria fagocitose por células epiteliais do intestino do hospedeiro, seguido de replicação e de transferência direta para outras células. O vinho é uma solução de composição complexa e tem atividade antimicrobiana comprovada devido, principalmente, ao seu conteúdo de etanol, ácidos orgânicos e compostos fenólicos. Este trabalho centrou-se em (i) comparar a suscetibilidade de estirpes de *L. monocytogenes* de origem alimentar e clínica à ação antimicrobiana do vinho, e (ii) na avaliação da influência do vinho sobre a capacidade de invasão de *L. monocytogenes* utilizando a linhagem de células Caco- 2 (células epiteliais intestinais humanas).

Foram utilizados 39 isolados de *L. monocytogenes*, 22 de origem alimentar e 17 de origem clínica pertencentes a diferentes serogrupos. Para medir o efeito de inativação do vinho tinto, cada isolado foi submetido a uma diluição de 1:10 de vinho, durante 120 segundos, a 25 ºC. Verificou-se que o vinho exerceu um forte efeito antilisterial, porém observou-se uma alta variabilidade fenotípica entre os isolados – os isolados clínicos mostraram ser significativamente mais resistentes ao vinho do que os isolados alimentares. Células viáveis de uma estirpe clínica (isolada a partir de um surto de listeriose que ocorreu em Portugal) recolhidas após a exposição ao vinho durante 15 seg evidenciaram uma maior capacidade de invadir a linha de células humanas Caco-2 em comparação com o controlo correspondente não exposto ao vinho. Este efeito não foi observado quando o teste de invasão foi realizado em dois outros isolados mais resistentes ao vinho. Tanto quanto é do nosso conhecimento, este é o primeiro estudo que utiliza um número substancial de isolados de *L. monocytogenes* de diferentes origens e com diversidade fenotípica e genética para estudar a sua suscetibilidade ao vinho. O trabalho também é inovador na avaliação da influência do vinho sobre as características de virulência de um organismo patogénio. Os resultados deste estudo evidenciam que o vinho tinto pode atuar como uma barreira ao crescimento e sobrevivência de *L. monocytogenes*, quando em contacto com alimentos contaminados.

Abstract

Listeria monocytogenes is a foodborne pathogen known for the severity of its infection, listeriosis, and for its capability of growing at harsh conditions such as refrigeration temperatures, wide pH range, low water activity and high salinity. *L. monocytogenes* can induce its own phagocytosis by the host's intestinal epithelial cells, followed by replication and direct transfer to other cells. Wine is a complex solution with proved antimicrobial activity due to its content of ethanol, organic acids and phenolics. This work focused on (i) comparing the susceptibility of food and clinical strains of *L. monocytogenes* towards wine, and (ii) on the evaluation of the influence of wine on the invasiveness ability of *L. monocytogenes* using the human intestinal epithelial cells Caco-2 cell line.

Thirty-nine isolates of *L. monocytogenes* were used in this study, 22 of food and 17 of clinical origin belonging to different serogroups. To measure the inactivation effect of red wine, each isolate was subjected to a 1:10 dilution of wine, during 120 seconds, at 25 ºC. Wine was found to exert an antilisterial effect, though a high phenotypic variability was observed among isolates - clinical isolates were found to be significantly more resistant to red wine than food isolates. Viable cells of one clinical strain (isolated from a listeriosis outbreak occurred in Portugal), collected after exposure to wine for 15 sec showed enhanced ability to invade the human intestinal Caco-2 cell line when compared to the corresponding unexposed control. This effect was not observed when the invasion test was done on two other isolates more resistant to wine. This is, to the best of our knowledge, the first study using a substantial number of *L. monocytogenes* strains from different origins and diverse phenotypic and genetic characteristics to study the susceptibility to wine. It is also innovative on assessing the influence of red wine on virulence traits of a foodborne pathogen. The results of this study give evidence that red wine can be seen as a significant barrier to the growth and survival of *L. monocytogenes* when in contact with contaminated food (food consumption scenario, for example).

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1 Introduction

1.1 *Listeria*

Listeria was named after the surgeon and pioneer of antisepsis Lord Lister in the 1860's (Ledermann, 2007). *Listeria* species are gram-positive, anaerobic facultative, nonsporulating, catalase positive, and oxidase-negative, rod-shaped bacteria of 0.4-0.5 x 1-2 μm with parallel sides and blunt ends. They are widely distributed in the environment: soil, water, vegetation, fresh and frozen poultry, animal feed, slaughterhouse wastes effluents, and feces from healthy animals and humans (Ludwig *et al*., 2001). They are capable of growing at pH 6-9, high salt concentrations (10% (w/v)), and temperatures ranging from 4 to 45 ºC but optimal growth occurs at 30 – 37 ºC; *Listeria* do not survive heating at 60 ºC for 30 minutes. The motility of this bacteria depends on its growth temperature, producing peritrichous flagella below 30 ºC and repressed at 37 ºC, the latter needed to achieve full virulence (Ludwig *et al*., 2001).

Listeria genus comprises 19 species: *L. aquatic, L. booriae, L. cornellensis, L. denitrificans, L. fleischmannii, L. floridensis, L. grandensis, L. grayi, L. innocua, L. ivanovii, L. marthii, L. monocytogenes, L. murrayi, L. newyorkensis, L. riparia, L. rocourtiae, L. seeligeri, L. weihenstephanensis, L. welshimeri* (Euzéby, 2015)*;* however, only *L. monocytogenes* and *L. ivanovii* are pathogenic affecting more than 50 animal species; furthermore, humans are only infected by *L. monocytogenes* (Zorn & Suárez, 2009; Bennett, 2015).

1.1.1 Listeria monocytogenes

L. monocytogenes was first described in 1926 by Murray *et al*. under the name of *Bacterium monocytogenes* because it caused fever and monocytes in their laboratory rabbits and guinea pigs. A year later, Pirie renamed it *Listerella hepatolytica* for the liver damage he found in gerbils and finally in 1940 he named it as this foodborne pathogen is currently known: *Listeria monocytogenes* (Farber & Peterkin, 1991; Ledermann, 2007).

Thirteen serotypes have been described for *L. monocytogenes*: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7, based on serological reactions between somatic (O factor) and flagella (H factor) antigens and their corresponding antibodies (Farber & Peterkin, 1991); however serotypes 1/2a, 1/2b, and 4b comprise the majority of the strains associated with human listeriosis cases and outbreaks (Orsi *et al*., 2011). This species is divided in four major

evolutionary subdivisions: lineage I (1/2b, 3b, 3c, 4b); lineage II (1/2a, 3a, 1/2c); lineage III (4a, 4b, 4c); and lineage IV (4a, 4b, 4c). Serotypes 1/2a, 1/2c, 1/2b, and 4b (lineages I and II) are associated with the majority human listeriosis cases (> 98%), while serotypes 4a and 4c (lineage III) are rarely associated with outbreaks despite their frequent isolation from a variety of food and environmental specimens (Orsi *et al*., 2011).

1.1.2 Growth conditions

L. monocytogenes differentiates from other bacterium for its capability of growing at hard conditions such as refrigeration temperatures (-0.5 – 9.3 ºC) (Walker *et al*., 1990); wide pH range (4.2 – 9.5) (Bover & Garriga, 2014) surviving lower values (pH 3.3 – 3.5) (Phan-Thanh & Montagne, 1998); low water activity (0.90 – 0.93); and high salinity 12 – 16% (w/v) NaCl (Bover & Garriga, 2014). These conditions make *L. monocytogenes* a serious hazard for the food industry, because it is widespread in the environment and is able to survive and grow in the food processing environment and ready-to-eat foods; furthermore, it can contaminate a wide range of products at different stages of production e.g. after pasteurization, due to its capacity to attach to abiotic surfaces and form biofilms (Alessandria *et al*., 2010).

1.1.3 Listeriosis

Listeriosis is the foodborne infection caused by *L. monocytogenes*. For the general population is a rare disease, when presented in healthy individuals is in the form of flu-like symptoms or as self-limited gastroenteritis; conversely, for the risk population (pregnant women and their fetus, newborns, the elderly and immunocompromised people) can be lethal with a fatality rate of 16 – 3 0% (Vázquez-Boland *et al*., 2001; Bortolussi, 2008; Bennett *et al*., 2015).

The infective dose and incubation period of *L. monocytogenes* is unknown but is estimated to be 10 – 100 million colony forming units (CFU) in healthy hosts, whereas for the risk groups it is of 0.1 – 10 million CFU (Bortolussi, 2008). The symptoms can develop any time from 2 to 90 days after consumption with a mean of 30 days (Bennett, 2015; Bortolussi, 2008).

Listeriosis is presented as non-invasive and invasive disease. As mentioned before, in healthy individuals the non-invasive disease presents as gastroenteritis accompanied by fever, watery diarrhea, nausea, headache, and pains in joints and muscles, the onset of the disease

usually occurs in a range of 6 hours to 10 days and usually lasts 1 to 3 days up to 7; in contrast, the invasive form comprises bacteremia, endocarditis and central nervous system (CNS) affections such as meningitis, encephalitis, rhombencephalitis, brain abscess and spinal cord infection (Bennett, 2015). Pregnant women are prone to develop bacteremia manifested as acute febrile illness, often accompanied by myalgias, arthralgias, headache, and backache; furthermore, the mother can transmit the disease to the fetus through the placenta resulting in stillbirth, spontaneous abortion or neonatal death, however, early antimicrobial treatment can result in the birth of a healthy infant (Bennett, 2015). For treatment, ampicillin is the preferred agent; when CNS infection or endocarditis are present the addition of gentamicin and trimethoprim-sulfamethoxazole are used. For people allergic to penicillin, trimethoprim-sulfamethoxazole is used (Allerberger & Wagner, 2010).

1.1.3.1 Invasion and spread

Listeriosis can be such a severe illness because *L. monocytogenes* can induce its own phagocytosis by host cells (nonphagocytic cells), followed by replication within those cells and direct transfer to another cell; then it can spread through the body protected from antibodies and complement. The immunity to *L. monocytogenes* is T cell mediated (Doyle 2001; Swaminathan *et al*., 2007). The bacterium starts by infecting the intestinal epithelial cells. From the intestine bacteria disseminate via the blood or lymph to the liver and spleen where most are killed by neutrophils acting with Küpffer cells. In people with inadequate T-cell mediated immune response, listeriae multiply in the hepatocytes and macrophages and is transported to infect other organs, particularly the brain and uterus (Doyle, 2001).

1.1.4 Virulence factors

Many factors are involved in all the steps of infection as shown in figure 1.1.

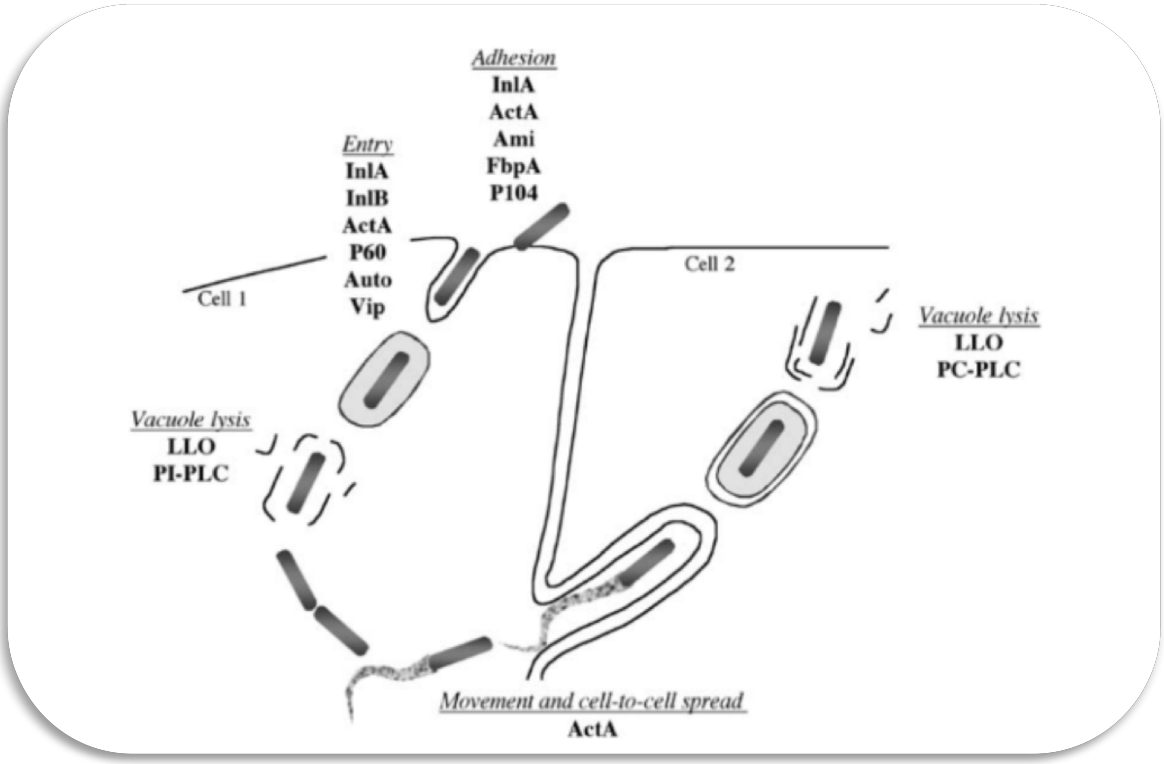

Figure 1.1 *Representation of the of infectious process of L. monocytogenes and the factors implicated in each step (in Swaminathan et al., 2007).*

Internalins A and B (InlA and InlB): are listeria surface proteins involved in the entry to host cells. IntlA binds to E-cadherin on the surface of host epithelial cells which stimulates the phagocytosis of *L. monocytogenes*. Similarly, InlB binds to Met receptor for the invasion of hepatocytes in the liver (Swaminathan *et al*., 2007).

Listeriolysin O (LLO): is a bacterial preforming toxin than enables the scape of *L. monocytogenes* from the vacuoles into the cytoplasm of the cell (Doyle, 2001).

Proteins P104 and P60: P104 is a surface protein involved in the adhesion to intestinal cells (Doyle, 2001). P60 is important in the immune response against listeriosis, because specific antibodies in immunocompetent individuals can prevent systemic infections (Swaminathan *et al*., 2007).

ActA protein: is a surface protein implicated in the attachment to cells and responsible for the actin-based motility of *Listeria*. It induces the polymerization of globular actin molecules to

form filaments along which the *Listeria* moves to adjacent cells without exposure to antibodies or other immunoactive molecules (Doyle, 2001).

Phospholipases: have a membrane-damaging activity and are involved in the escape from phagosomes (Swaminathan *et al*., 2007). Two are produced by *L. monocytogenes* phosphatidylinositol-specific phospholipase C (PI-PLC) and a broad-range or phosphatidylcholine-specific phospholipase C (PC- PLC). PI-PLC aids in escape from the primary vacuole while PC-PLC is active during cell-to-cell spread of bacteria and it can substitute LLO (Doyle, 2001).

Metalloprotease: PC-PLC is produced as an inactive precursor, to activate it a bacterial zinc-dependent metalloprotease and a host cell cysteine protease are required (Doyle, 2001).

Vip: is a virulence gene that encodes the LPXTG surface protein required for entering mammalian cells at intestinal level and later stages of the infection (Swaminathan *et al*., 2007).

Clp proteases (caseinolytic proteins) and ATPases: ClpC ATPase is a general stress protein that assists in the disruption of the vacuolar membrane. ClpC also modulates expression of the ActA protein and the internalins. ClpP serine protease is required for growth under stress affecting the activity of listeriolysin O, also ClpE, is involved in listerial pathogenesis (Doyle, 2001).

1.1.5 Human cases and outbreaks of listeriosis

According to the European Food Safety Authority (EFSA) latest European Union (EU) summary report on zoonoses, zoonotic agents and foodborne outbreaks for 2014, 27 member states confirmed a total of 2,161 cases of human listeriosis with a notification rate of 0.52 cases per 100,000 population representing an increase of 30% compared with 2013. For the period 2008 – 2014 the increasing trend of listeriosis was statistically significant. Of all the zoonotic diseases under EU surveillance, listeriosis caused the most severe human disease with 98.9% hospitalizations and 210 deaths, representing a case-fatality rate of 15% (out of the 1,401 confirmed cases with known outcome). The highest number of deceased was reported by France: 51 cases. Regarding outbreaks of listeriosis, there were several small but

Denmark reported a large outbreak comprising 41 cases; on the other hand, Sweden who in 2013 had an outbreak involving 50 cases, in 2014 only 27 cases were reported (EFSA, 2015).

1.2 Wine

Wine is a complex solution of a vast number of chemicals e.g. 160 esters have been identified, despite that individually they are found in concentrations below human detection (10^{-4} - 10^{-9} g/L) together they are important for the organoleptic characteristics (Jackson, 2014). The aromatic compounds are found in 0.8 – 1.2 g/L mainly as fusel alcohols (50% of all volatile substances), volatile acids and fatty acid esters. Carbonyls, phenols, lactones, terpenes, acetals, hydrocarbons, and sulfur and nitrogen compounds are present in much smaller concentrations but their importance lays in the varietal and sensory features conferred to the wine's fragrance (Jackson, 2014).

The main components of wine are water and ethanol (approximately 98%); followed by trace components (vitamins, sugars, nitrogenous components, cations and anions), volatiles (fusel alcohols, esters, ketones, C13 norisoprenoids, fatty acids, phenols, amides, other) and acids (tartaric and malic), representing 1%, 0.5% and 0.5% respectively (Jackson, 2014). Wines can be classified in two major groups: table wines whose alcohol content is below 14% and dessert wines produced from grapes high in sugar and low in acid content (Friedman, 2014). The general composition of red and white wines for both categories is shown in table 1.1 where the content of phenols between red and white wines is noted as the major difference in both table and dessert wines.

Table 1.1 *Estimates of typical gross composition (% weight) of wines (in Soleas et al. 1997)*

	Table wines		**Dessert wines**	
Component	White	Red	White	Red
Water (by difference)	87	87	76	74
Ethanol	10	10	14	14
Other volatiles	0.04	0.04	0.05	0.05
Extract	2.6	2.7	10.1	12.2
Sugars	0.05	0.05	8	10
Pectins	0.3	0.3	0.25	0.25
Glycerol	1.1	1.1	0.9	0.9

Acids	0.7	0.6	0.5	0.05
Ash	0.2	0.2	0.2	0.2
Phenols	0.01	0.2	0.01	0.1
Amino acids	0.25	0.25	0.2	0.2
Fats, terpenoids	0.01	0.02	0.01	0.02
Vitamins, etc.	0.01	0.01	0.01	0.01
Total	100	100	100	100

1.2.1 Antimicrobial properties of wine

Wine is active against bacteria (human and plants), fungus, protozoans and several viruses, including herpes simplex virus, poliovirus, hepatitis A, as well as in common cold viruses (rhinoviruses and coronaviruses) (Jackson, 2014; Muñoz-González, *et al*. 2014; Friedman, 2014; Cueva *et al*., 2010; Király-Véghely *et al*., 2009). Takkouche *et al*. (2002) found an inverse association between moderate wine drinkers and the incidence of common cold. As stated before wine is a complex solution with numerous components showing antimicrobial properties, the mechanisms behind this are not well understood: low pH (3.0 to 4.0), high organic acid content (titratable acidity ≥6.0 g/L tartaric acid), relatively high ethanol (10% to 15%), and potentially high total sulfur dioxide (0 to 300 ppm) are contributors (Waite & Daeschel, 2007).

The concentrations of alcohol found in wine are not high enough to be fully responsible for the antimicrobial action, rather the interaction of different constituents, for example, the modification of anthocyanins during fermentation increases their toxicity to viruses, protozoans, and bacteria (Gram positive and negative) (Jackson, 2014); as Boban *et al*., (2010) found the antimicrobial activity cannot be attributed to the phenolic or nonphenolic constituents of wine, nor based on its components predict the antimicrobial activity of a wine. Furthermore, Møretrø & Daeschel (2006) evaluated wine components individually and combined concluding that the synergistic effect of organic acids, ethanol, and low pH seems to be responsible for a major part of the antibacterial effect. Table 1.2 shows selected studies on the effects of wine against foodborne pathogens. All the studies used red wine with the exception of Møretrø & Daeschel (2006) who reported that red wine was more effective than white for the tested bacteria, and Liu *et al*. (2006) who found no significant differences between red and white wines in the inactivation of *Vibrio parahaemolyticus*; furthermore no significant differences were found between those wines with and without added sulfite.

Table 1.2 *Studies on the antimicrobial effect of wine against foodborne pathogens.*

Pathogen	Objective	Findings	Reference
Bacillus cereus	To evaluate the antimicrobial activity of wine against *B. cereus* vegetative cells and spores.	i)Wine inactivated *B. cereus* cultures to undetectable levels in <10 s, the time increased as wine was diluted, ii) spores were highly resistant to inactivation, iii) phenolic compounds were inactive against vegetative cells, iv) drinking wine during a meal may lower the risk of toxi-infection by reducing the number of vegetative cells in the stomach and the germination of spores in the small intestine.	Vaz *et al.* (2012)
Campylobacter jejuni	To characterize the effect of exposure to wine on the survival of *C. jejuni*. This characterization aimed to describe the effects of this exposure in both direct-immersion, marinade conditions and in simultaneous-consumption (wine/food/bacteria) scenarios.	i) Red wine inactivated the foodborne pathogen within 30 s, ii) ethanol combined with organic acids (malic, tartaric) acted synergistically with an inactivation similar to wine itself, iii) the results from simulated consumption scenarios in a model stomach, suggest that ingestion of wine with food significantly decreases the number of *C. jejuni* persisting further in the alimentary tract, iv) suggests that immersion of foods in wine, e.g. marinades lowers the risk of of cross contamination of cooked foods with this pathogen.	Carneiro *et al.* (2008)
Escherichia coli, Proteus mirabilis, Serratia marcescens, Flavobacterium sp. and Klebsiella pneumoniae (human origin*); E. coli* ATCC35218, *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC25923, *S. aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853	To investigate the antimicrobial properties of pure phenolic compounds (flavonoids and phenolic acids) and total polyphenols of different Argentinean wines, Cabernet Sauvignon, Malbec and Merlot against food borne pathogens that are widely distributed in the environment and frequently detected in fresh and processed foods.	i) All wine samples showed antimicrobial properties, ii) inhibition increased as the polyphenols concentration of wines increased, iii) clarified wines were inactive against all bacteria, indicating that polyphenolic compounds present in red wines, are responsible for the antimicrobial effects observed	Rodríguez Vaquero *et al.* (2007)
Escherichia coli O157:H7 and *Salmonella* spp	To determine if wine has significant antimicrobial effects on foodborne enteric pathogens. The specific aims of this research were to (1) determine the survival of *E. coli* O157:H7 and *Salmonella* in red and	i) Consuming wine with a meal may protect against some food poisoning organisms such as *S. typhimurium*, but not against others such as *E. coli* O157:H7 ii) both *Salmonella* and *E. coli* O157:H7 are rapidly inactivated in wine and this effect may be enhanced with an increased acid content iii) *Salmonella* and *E. coli* O157:H7 were found to survive up to 16 d in grape juice.	Just & Daeschel (2003)

	white wine and grape juice and (2) determine the effect of wine on *E. coli* O157:H7 and *Salmonella* survival in a model stomach system		
Salmonella enteritidis, *Escherichia coli* O157:H7, and *Vibrio parahaemolyticus*	Examine the antibacterial activity against the three entero-pathogenic bacteria *in vitro* and *in vivo*. To identify which fraction of wine had antibacterial activity, and examine the ability of wine and the fraction to protect against infection *in vivo*.	i) Red and white wines have antibacterial activity against the 3 entero-pahtogens killing them within 30 minutes, ii) the evaporated fraction contained in wine seemed responsible for this and not polyphenols, iii) when tested in mice, neither red or white wine showed prevention properties against *S. enteritidis* wether given "one shot" treatment or continoues administration, iv) the antibacterial property seems to be limited to *in vitro* circumstances.	Sugita-Konishi *et al.* (2001)
Listeria innocua	To study the the bactericidal effect of wine on *L. innocua* (surrogate for *L. monocytogenes*) in a food matrix, under simulated gastric conditions (stomach model), and to evaluate specific influence of some wine components on this effect.	i) Red wine volumes, equivalent to the ingestion of one glass and half a bottle, induced a 3–4 log CFU reduction of the initial pathogen count in <2 h, ii) the combination of ethanol and malic and tartaric acids was less effective than wine, iii) the kinetics of the bactericidal effect of red and white wine, differed demonstrating that the antimicrobial effects of wines depends on composition.	Fernandes *et al.* (2007)
L. monocytogenes, Escherichia coli O157:H7, *Salmonella typhimurium,* and *Staphylococcus aureus*	To test the antibacterial effect of wine against wild-type strains and sigma mutants of the pathogens *E. coli O157:H7, L. monocytogenes, S. typhimurium,* and *Staphy lococcus aureus*. The antibacterial effects of selected components of wines were also tested individually and in combination. Moreover, the protection of the bacteria against wine by stress dependent protection systems was evaluated.	i) Red and white had bactericidal activity against all strains, red wine was more effective than white, ii) of the wild-type strains, *S. Typhimurium* was the most sensitive to wine, followed by *E. coli* O157:H7, *L. monocytogenes*, and *S. aureus*, iii) the alternative sigma factors seemed to be involved in protection of the bacteria against wine, mutants (having this factor disrupted) were generally more sensitive to wine than their wild-type counterparts, iv) preincubation of *E. coli* O157:H7 and *S. aureus* (wild-type) in sublethal concentrations of wine and ethanol and pH 4.5 did not increase their tolerance against wine or against the mixture of organic acids and ethanol, v) the synergistic effect of organic acids, ethanol, and low pH seems to be responsible for a major part of the antibacterial effect of wine (the composition of 0.15% malic acid, 0.6% tartaric acid, 15% ethanol, and pH 3.0 had the strongest bactericidal effect).	Møretrø & Daeschel (2006)
Salmonella enterica serovar Enteritidis and *Escherichia*	To examine antibacterial activity of the intact red wine and	i) Antibacterial activity of the samples was: intact wine > phenols-stripped wine > dealcoholized wine > combination of ethanol	Boban *et al.* (2010)

coli	its derivatives under the same experimental conditions against 2 common foodborne pathogens, *S. enterica* serovar Enteritidis and *E. coli*. To separate the role of wine phenolics, ethanol, and pH from other wine constituents, the antimicrobial effects of intact wine were compared to that of phenols-stripped wine, dealcoholized wine, ethanol, and low pH applied separately and in combination.	and low pH > low pH > ethanol, ii) low pH and ethanol had synergistic effect, whereas individually their antibacterial activity is negligible, iii) antibacterial activity of the samples could not be related to their total phenolics and resveratrol content, antioxidant capacity, ethanol content, or pH, iii) antimicrobial activity of complex solutions such as intact wine cannot be exclusively attributed to its phenolic or nonphenolic constituents, nor can the antimicrobial activity of wine be predicted on the basis of its particular components.	
S. aureus and *E. coli* O157:H7.	To look at 4 wine parameters, pH, titratable acidity, sulfur dioxide concentration, and ethanol concentration, in various combinations within a wine background to evaluate antimicrobial activity against the food-borne pathogens *S. aureus* and *E. coli* O157:H7.	i) pH was found to be the most critical factor in predicting inactivation of both *S. aureus* and *E. coli* O157:H7, ii) Molecular sulfur dioxide, titratable acidity, and ethanol concentration also contributed to the inactivation of *S. aureus*. Ethanol concentration was also found to contribute the efficacy of wine treatments on *E. coli* O157:H7. iii)Total sulfur dioxide and free sulfur dioxide were not predictive of wine efficacy against either pathogen tested. These findings indicate the importance of each parameter in wine to be used for potential disinfection purposes.	Waite & Daeschel (2007)
Vibrio parahaemolyticus	To investigate the antibacterial activities of both red and white wines against *V. parahaemolyticus* in laboratory-contaminated oysters and compared the bactericidal effects of wines with and without added sulfites on inactivating *V. parahaemolyticus*.	i) The populations in wine-treated whole oysters decreased by >1.7 and >1.9 log MPN/g after 24 h at 7 and 25 ºC, respectively; no significant differences were found between red and white wines or between wines with and without added sulfite, ii) both red and white wines were more effective in inactivating *V. parahaemolyticus* in oyster meat homogenate populations decreased rapidly (a 3.89- log MPN/g reduction) to nondetectable levels after 30 min at 25 ºC, iii) These results suggest that chewing oysters before swallowing when eating raw oysters may result in greater inactivation of *V. parahaemolyticus* if wine is consumed	Liu *et al*. (2006)

1.2.2 Wine compounds contributing to its antimicrobial properties.

Several studies, evaluating wine as a whole or specific groups of compounds, have identified the major constituents with antimicrobial effects, which are described below.

1.2.2.1 Ethanol

The efficacy of ethanol as antiseptic is increased in the presence of water and its antimicrobial activity is optimum in the range of 60 – 90%, causing membrane damage by solubilizing lipids and denaturating proteins with subsequent interference with metabolism and cell lysis (McDonnell & Russell, 1999; Barker & Park, 2001), thus the concentrations of ethanol present in wine are not high enough to be fully responsible for the antimicrobial action of wine since the effects of wine are greater than the same concentration of diluted ethanol (Møretrø & Daeschel, 2006). This is consistent with Boban *et al*. (2010) findings who reported that low pH and ethanol had a synergistic effect, whereas individually their antibacterial activity is unimportant.

1.2.2.2 Organic Acids

Acidity in wine is divided into volatile and fixed, meaning the acids that can be readily removed by steam distillation and those that are poorly volatile, respectively; both make up the total acidity. Fixed acidity in wines can vary from less 2 g/L to over 5 g/L (Jackson, 2014). The main organic acids of wine are tartaric and malic, for the ones undergoing malolactic fermentation malic acid is metabolized to lactic acid. Fixed acidity is responsible for the antimicrobial effect of organic acids because it confers low pH to the wine in which most bacteria do not grow and the fatty acids remain undissociated. In a study evaluating the antimicrobial activity of wine against *Bacillus cereus* vegetative cells and spores Vaz *et al*. (2012) found that organic acids contribute to the antimicrobial effect of wine; additionally, they strengthen the action of phenolics and ethanol. Similarly, Møretrø & Daeschel (2006) found a higher reduction in viable cells of *S. aureus, L. monocytogenes, S. typhimurium* and *E. coli* in a mixture of ethanol, malic and tartaric acid (pH 3) than when each was tested individually. Carneiro *et al*., (2008) used a the mixture of tartaric, acetic, lactic and citric acids combined with ethanol in concentrations found in wine and their combination showed higher bactericidal effect than the mixture of acids and ethanol separately. The effectiveness

of organic acids is related to pH which determines the degree of dissociation of the acid. The undissociated organic acids cross the cell membrane lipid bilayer more easily. Once inside the cell, the acids dissociate because of the higher intracellular pH (near neutrality), releasing protons that acidify the cytoplasm which suppress cell enzymes affecting its metabolism. In order to restore the optimal intracellular pH, protons need to be pump out by the H^+-ATPase demanding considerable metabolic energy in the form of adenosine triphosphate (ATP) that could lead to depletion of cellular ATP with the eventual cell death due to energy depletion; furthermore, the remaining anions in the cytoplasm can inhibit the synthesis of macromolecules, enzyme activity, nutrient transport systems within the cytoplasm resulting in cell death (Swaminathan *et al*., 2007; Ng & Koh, 2016).

1.2.2.3 Phenolic compounds

Phenolics or phenols are a complex group comprising a huge amount of compounds important for the quality (appearance, taste, mouth-feel, fragrance) of wine and its antimicrobial properties. The main source is the grape (skin, seed and stems) followed by smaller amounts that may be extracted from oak barrels, finally yeasts produce trace amounts during fermentation (Jackson, 2014). Chemically, phenols are cyclic benzene compounds with one or more hydroxyl groups associated directly with the ring structure (Soleas *et al*., 1997). Concentration of phenols is expressed as gallic acid equivalents (GAE) based on their chemical reducing capacity relative to the equivalent reducing capacity of that component. For red wines concentration ranges from 1800 to 4059 GAE mg/L (average 2567 GAE mg/L), and 165 to 331 GAE mg/L (average 239 GAE mg/L) for white (Frankel *et al*., 1995).

The main phenolics found in wine are: flavonoids (two phenyl groups) whose polymers are tannins, and non-flavonoids (containing one phenyl group).

<u>Flavonoids</u>

Flavonoids characterize red wines more than any other feature, accounting for more than 85% of their phenolic content (≥1000 mg/L), conversely in white wines they constitute less than 20% of the total phenolic content (≤50 mg/L) (Jackson, 2014). The rest consists mainly of the non-flavonoid, caffeic acid. The amount of flavonoids in wine is influenced by a number of factors starting from the grape production (cultivar, vintage, climatic conditions of the region) to the vinification process (temperature, length of skin contact, mixing, type of fermentation/aging). This family of compounds share a common C6-C3-C6 skeleton

consisting of two phenolic rings (named A and B) linked together by a heterocyclic pyran ring (C-ring) (Terrier *et. al.*, 2009).

The most common flavonoids in wine are flavonols, catechins (flavan-3-ols) and anthocyanins (in red wines), all of them are either free or polymerized with other flavonoids, non-flavonoids or both. There are also small amounts of free leucoanthocyanins (Soleas *et al.*, 1997).

Flavanol oligomers and polymers are also called condensed tannins or proanthocyanidins for their capacity to precipitate proteins and to release anthocyanidin when heated in acidic conditions (Terrier *et. al.*, 2009). Anthocyanidins are the sugar-free counterparts of anthocyanins and five are identified in wine: delphinidin, cyanidin, petunidin, peonidin and malvidin (Monagas & Bartolomé, 2009).

Non-Flavonoids

Non-flavonoid phenolic constituents in wine are divided into hydroxybenzoic acids (HBA), hydroxycinnamic acids (HCA), volatile phenols, stilbenes and miscellaneous compounds (e.g. lignans and coumarins). They stabilize the color of red wines despite they are colorless, contribute to flavor and some of them exhibit potent biological activities (Rentzsch *et al.*, 2009).

The most common derivatives from HBA found in wine are gallic acid, gentisic acid, *p*-hydroxybenzoic acid, protocatechuic acid, syringic acid, salicylic acid, and vanillic acid, and from HCA are caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid in *cis*- and *trans*-forms, the latter is more prevalent due to its stability (Rentzsch *et al.*, 2009).

The nature of non-flavonoids is influenced by the material the wine is aged in; for wines not aged in oak the derivatives of HCA are higher than those of HBA; whereas in wines aged in oak, levels of HBA derivatives (especially ellagic acid) are higher (Soleas *et al.*, 1997).

Stilbenes are synthesized in the grape as response to stress and are known for their antioxidative, anticarcinogenic and antimutagenic potency; one of the most comprehensively studied is resveratrol. Resveratrol exists in the *cis*- and *trans*- isomers, as in the ß-glucoconjugated form. The 3-O-ß-D-glucosides of *cis*- and *trans*- resveratrol as cis-and trans-configured resveratrol are called piceids. The oligomeric and polymeric forms of stilbenes are called viniferins (Rentzsch *et al.*, 2009).

In wine ε-viniferin, δ-viniferin, pallidol (resveratrol dimer), α-viniferin, (resveratrol trimer) and hopeaphenol (resveratrol tetramer) have been identified. Free *trans*- and *cis*-

resveratrols are present in a concentration range of 0.2–13 mg/L in red wines and 0.1–0.8 mg/L in white wines; for resveratrol-3-*O*-glucoside, concentrations range from 0.3–9 mg/L in red and 0.1–2.2 mg/L in white. In comparison to wine, grapes contain mainly *trans*-resveratrol glucoside in concentrations ranging from 1.5 to 7.3 μg/g (Rentzsch *et al*., 2009).

Phenolic compounds have been extensively studied for the health benefits aforementioned. Rodríguez Vaquero *et al*. (2007) studied the antimicrobial properties of four phenolic acids (gallic, vanillic, protocatechuic and caffeic acid) and three flavonoids (rutin, catechin and quercetin) of different wines and found that as their concentration increased so did the bactericidal effect of wine, furthermore the same wines after clarification (controls) showed no inactivation, relating directly the antimicrobial effects to the polyphenolic compounds; in contrast Vaz *et al*. (2012) found that resveratrol, ferulic acid, p-coumaric acid, kaempferol and quercetin were inactive against *B. cereus* vegetative cells. Boban *et al*. (2010) found that after wine, their phenols-stripped counterparts where most effective thus the antimicrobial activity of wine cannot be exclusively attributed to its phenolic or nonphenolic constituents; likewise, red wine with the highest resveratrol concentration had the highest inhibitory effect on *Helicobacter pylori* urease activity (virulence factor) however, resveratrol from wine required lower concentrations than the pure compound to produce the same results, probably due to synergic reactions with the other wine constituents (Paulo *et al*., 2011).

1.3 Objective

The bactericidal effect of wine on *L. innocua (surrogate* of *L. monocytogenes)* has already been shown in model stomach systems. This work further characterized the activity of wine against *L. monocytogenes* comparing the sensitivity of food strains with clinical strains. Another major aim of this work was to evaluate the effect of sub-lethal levels of wine on the capacity of *L. monocytogenes* to invade host cells which is the first step of the infective cycle. Human intestinal epithelial cells (Caco-2 cell line) were used to assess the influence of wine on the invasiveness ability of *L. monocytogenes*.

2 Materials and methods

2.1 *L. monocytogenes* isolates

Thirty-nine isolates of *L. monocytogenes* were used in this study (table 2.1). All the isolates belong to the *Listeria* Research Center of Escola Superior de Biotecnologia (LRCESB) (Porto, Portugal) and were selected to include two different origins: clinical and food. These isolates have been previously characterized by serogroup multiplex-PCR, pulsed-field gel electrophoresis (PFGE), and antibiotic resistance (Komora *et al*., 2016).

Table 2.1 List of *food and clinical isolates of L. monocytogenes selected for this study*.

Origin	Isolate code	Serogroup[a]	PFGE type[b]	Antibiotic Susceptibility[c]
Food	Lm 654	IVb	263	$ERY^{S}NIT^{S}CIP^{S}$
	Lm 864/4	IVb	133	$ERY^{R}NIT^{S}CIP^{S}$
	Lm 830/1	IVb	133	$ERY^{R}NIT^{R}CIP^{S}$
	Lm 841/2	IVb	133	$ERY^{R}NIT^{R}CIP^{S}$
	Lm 1162	IVb	17	$ERY^{S}NIT^{R}CIP^{S}$
	Lm 1604/2	IVb	83	$ERY^{S}NIT^{S}CIP^{S}$
	Lm 1728	IVb	79	$ERY^{S}NIT^{R}CIP^{S}$
	Lm 1940/1	IVb	ND	$ERY^{S}NIT^{S}CIP^{R}$
	Lm 949	IIb	232	$ERY^{S}NIT^{S}CIP^{R}$
	Lm 969/3	IIb	151	$ERY^{S}NIT^{R}CIP^{S}$
	Lm 971	IIb	246	$ERY^{S}NIT^{S}CIP^{R}$
	Lm 1043	IIb	16	$ERY^{S}NIT^{R}CIP^{S}$
	Lm 800/2	IIb	145	$ERY^{R}NIT^{R}CIP^{S}$
	Lm 1216	IIb	278	$ERY^{S}NIT^{S}CIP^{S}$
	Lm 1382/1	IIb	147	$ERY^{S}NIT^{R}CIP^{S}$
	Lm 1486/1	IIb	10	$ERY^{S}NIT^{R}CIP^{S}$
	Lm 1535	IIb	156	$ERY^{S}NIT^{R}CIP^{S}$
	Lm 925/1	IIb	43	$ERY^{S}NIT^{S}CIP^{S}$
	Lm 1852/3	IIb	ND	$ERY^{S}NIT^{R}CIP^{S}$
	Lm 1846/1	IIb	ND	$ERY^{S}NIT^{R}CIP^{S}$
	Lm 863/1	IIc	129	$ERY^{S}NIT^{S}CIP^{S}$
	Lm 1305	IIc	206	$ERY^{S}NIT^{S}CIP^{S}$

	Lm 2104	IVb	86	$ERY^{S}NIT^{R}CIP^{S}$
	Lm 2264	IVb	53	$ERY^{S}NIT^{S}CIP^{R}$
	Lm 2265	IVb	53	$ERY^{S}NIT^{S}CIP^{R}$
	Lm 2542	IVb	70	$ERY^{S}NIT^{S}CIP^{S}$
	Lm 2571	IVb	53	$ERY^{S}NIT^{S}CIP^{R}$
	Lm 3390	IVb	393	$ERY^{S}NIT^{R}CIP^{R}$
	Lm 1543	IVb	54	$ERY^{S}NIT^{S}CIP^{S}$
	Scott A	4b	ND	ND
Clinical	Lm 2103	IIa	9	$ERY^{S}NIT^{S}CIP^{R}$
	Lm 2388	IIa	96	$ERY^{S}NIT^{S}CIP^{R}$
	3391	IIa	332	$ERY^{S}NIT^{R}CIP^{R}$
	2086	IIa	36	$ERY^{S}NIT^{S}CIP^{S}$
	EGDe	1/2a	ND	ND
	2065	IIb	37	$ERY^{S}NIT^{S}CIP^{S}$
	CLIP 21369	1/2b	ND	$ERY^{S}NIT^{S}CIP^{R}$
	2658	IIb	87	$ERY^{S}NIT^{R}CIP^{R}$
	1062	IIb	42	$ERY^{S}NIT^{R}CIP^{S}$

[a] Determined by Multiplex-PCR that differentiates major serogroups IVb (includes serotypes 4b, 4d, and 4e), serogroup IIa (includes serotypes 1/2a and 3a), and serogroup IIb (serotypes 1/2b, 3b, and 7); except for reference strains Scott A, EGDe, and CLIP 21369 with known serotypes.

[b] Characterization by pulsed-field gel electrophoresis (PFGE) enzimes *Asc*I and *Apa*I

[c] ERY - erythromycin, minimum inhibitory concentration (MIC) ≥ 4 μg/mL; NIT - nitrofurantoin, MIC ≥ 128 μg/mL; CIP - ciprofloxacin, MIC ≥ 8 μg/mL; R resistant; S susceptible

ND – not determined

2.2 Storage conditions

Stock cultures of *Listeria* strains were kept in tryptic soya broth with yeast extract 0.6% w/v (TSBYE, Lab M, Heywood, Lanchashire, UK) supplemented with 30% (v/v) of glycerol at -80 °C.

2.3 *L. monocytogenes* inoculum preparation

Before use, frozen stocks were streaked onto brain heart infusion (BHI, Biokar Diagnostics, Beauvais, France) agar plates and incubated at 37 °C overnight. One single colony of each isolate was transferred into 5 mL of BHI (Biokar) and incubated at 37 ºC for 24 h. The cultures were then sub-cultured in 11 mL of BHI (0.1% v/v) and incubated at 37 ºC for 18 h to reach the stationary phase. The cells were washed by centrifugation (10,000 x g, 5

min, 4 ºC; Rotina 35R, Hettich, Germany) in sterile phosphate-buffered saline (PBS; pH = 7.4) and cell pellet was suspended in the same volume of PBS to obtain an inoculum concentration of approximately 10^9 colony forming units (CFU)/mL. This procedure was done immediately before the experiments and 100 μL of each sample were collected to obtain the initial cell count.

2.4 Wine sterilization

Alandra red wine (2014, Alentejo region, Portugal) with 13% of ethanol (v/v) was used. The wine was filter sterilized using 0.45 μm - 25 mm cellulose acetate membranes (Frilabo sterile syringe filters, USA) and kept at 4 °C in sterile Scott Duran flasks of 100 mL until used.

2.5 The inactivation effect of wine *on L. monocytogenes*

For each isolate, inoculum aliquots of 2.5 mL were added to 22.5 mL of diluted wine (1:10 in sterile deionized water) pre-warmed at 25 ºC during 30 min in a thermostated water bath (Julabo SW22, Seelbach, Germany) before inoculation to allow temperature equilibration. At defined time points (15, 30, 60 and 120 seconds after inoculation) 100 μL of sample were collected, and vortexed for homogenization. As a control, for each isolate, 2.5 mL of inoculum were added to 22.5 mL of sterile PBS that was kept at 25 ºC and 100 μL of sample were collected at 2 time points (0 and 120 seconds). The experiments and corresponding controls were conducted in duplicate.

2.6 Bacterial enumeration

Samples were serially diluted in sterile PBS, and the dilutions subsequently plated on BHI agar plates in duplicate by the drop count technique (Miles & Misra, 1938). After incubation at 37 ºC for 24 h the colonies were counted, and the CFU/mL calculated. Microbial counts were transformed to logarithmic reduction using the equation: log (N/N0), where N is the microbial cell density at a particular sampling time and N0 is the initial cell density.

2.7 Caco-2 Invasion assays

Two food and two clinical strains were selected for this assay; selection was preformed to include from each origin the isolate showing higher resistance to wine and the strain more sensitive to wine.

2.7.1 Growth of *L. monocytogenes* for Caco-2 invasion assays.

Resistant *L. monocytogenes* strains (1852/3 and 2658) were grown and exposed to diluted wine as described above. At 15 sec exposure, 25 mL of sterile PBS was added and cells were immediately centrifuged (7000 x g, 5 min, 4 ºC), washed once with PBS and resuspended in 4 mL of PBS. Susceptible strains (969/3 and 2542) were grown and exposed to diluted wine as described above, however due to the considerable decrease in cell numbers after 15 seconds (3.7 log reduction for 969/3 and 2.4 log reduction for 2542), and to guarantee enough cell numbers for the invasion assay, the experiment to the wine exposure was performed using 10 times more volume of diluted wine (i.e. 250 mL) and inoculum (i.e. 25 mL). After 15 sec, 250 mL of sterile PBS was added, and each suspension was immediately centrifuged (7000 x g, 15 min, 4 ºC), and washed once with PBS. Cell-free supernatant was discarded and the pellet was resuspended in 2.5 ml of fresh PBS. As a control, for each invasion assay, the four *L. monocytogenes* strains were grown and treated in the same exact conditions, however, diluted wine was replaced by PBS. Furthermore, for sensitive strains controls, in the last step, the pellet was resuspended in the same initial volume of PBS instead of 2.5 mL (otherwise the initial cell numbers for the invasion assay would be exceedingly high).

2.7.2 Tissue culture invasion assays.

Caco-2 invasion assays were performed as previously described by Nightingale *et al*. (2005) with minor adjustments. The tumor-derived human colorectal epithelial cell line Caco-2 was grown in T75 flasks using Eagle's minimal essential medium (EMEM) (Lonza, Verviers, Belgium) containing 20% fetal bovine serum (FBS) (Lonza), 1% sodium pyruvate (Lonza) and 1% non-essential amino acids (Lonza), and incubated at 37 ºC with 7% (v/v) CO_2 atmosphere. For invasion assays, $5.0x10^4$ Caco-2 cells were seeded into 24-well plates (Costar, Corning, NY, USA) in EMEM and incubated for 48 h at 37 °C. Caco-2 monolayers were subsequently inoculated with 10 μL of the *L. monocytogenes* suspension treated as

detailed above and incubated at 37 °C for 30 min. The inoculums were immediately serial diluted and plated on BHI agar plates. Each well was then washed three times with 1 ml of sterile PBS to remove any unattached, extracellular *L. monocytogenes*. Subsequently, infected cells were incubated with 1 ml of pre-warmed fresh EMEM and at 45 min post-inoculation, the medium was replaced with fresh Caco-2 medium containing 150 μg/ml gentamicin (Gibco BRL, Gaithersburg, MD) in order to kill remaining extracellular bacteria. At 90 min post-infection, the medium was aspirated and the wells were washed three times with sterile PBS. Caco-2 cells were lysed by the addition of 500 μL of ice-cold sterile ultra-pure water and vigorous pipetting. Lysed Caco-2 cell suspensions were collected, serial diluted in sterile PBS and plated by the spread plating method on BHI agar plates, which were incubated at 37 °C for 24 h for determination of bacterial counts. The invasion efficiency was reported as the percentage of the inoculum recovered by the enumeration of intracellular bacteria. An uninoculated BHI broth were included as controls in each invasion assay. Three independent invasion assays were performed for each strain.

2.8 Statistical Analysis

One-way analysis of variances (ANOVA) was carried out to assess statistically significant differences among isolates. All tests were performed to a 5% significance level using IBM SPSS® Statistics® 20 for Windows® (SPSS Inc., Chicago, USA).

3 Results and discussion

3.1 Effect of wine on the survival of *Listeria monocytogenes*

In this study we tested the resistance of 17 clinical and 22 food *L. monocytogenes* isolates to red wine (1:10 dilution) during 120 sec, at 25 ºC. These experimental conditions (i.e. wine dilution factor, temperature and time) were selected based on preliminary experiments where non diluted wine and dilutions 1:2, 1:4 and 1:8 dilutions at 37 ºC exerted a very strong lethal effect, dropping viable cell counts to no detectable levels in less than 15 sec (data not shown). Immediately after 15 sec exposure a high variability among isolates was observed (figure 3.1), with log reduction values ranging from -0.4 to -2.4 for clinical isolates and from -0.5 and -3.7 for food isolates. Within the time of the experiment a substantial decrease in cell viability occurred (figures 3.2 – 3.3); after 120 sec exposure time, 11 food (50%) and 4 clinical (23.5%) isolates suffered reductions higher than 4.5 log cycles (data not shown), i.e. cell counts below or at the detection limit of the enumeration technique (<500 CFU/mL).

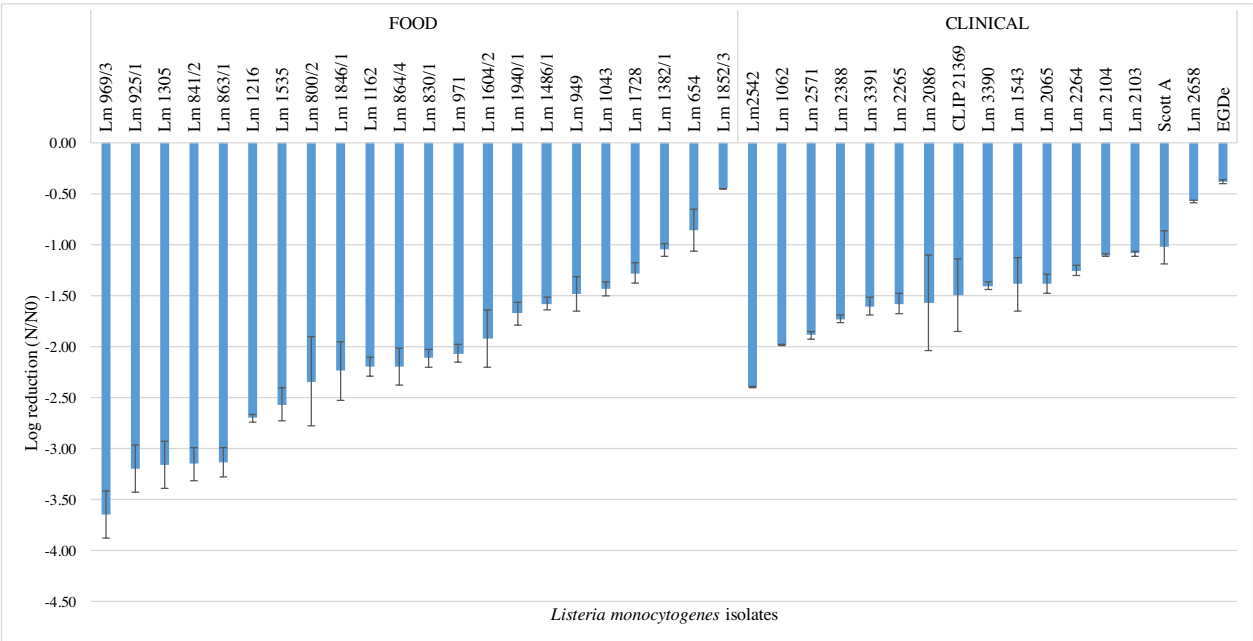

Figure 3.1 *Log reduction of food and clinical isolates of L. monocytogenes strains after 15 sec exposure to 1:10 dilution of red wine, rank-ordered according to their susceptibility. Data represent mean of duplicate assays and error bars represent the standard deviation of the mean.*

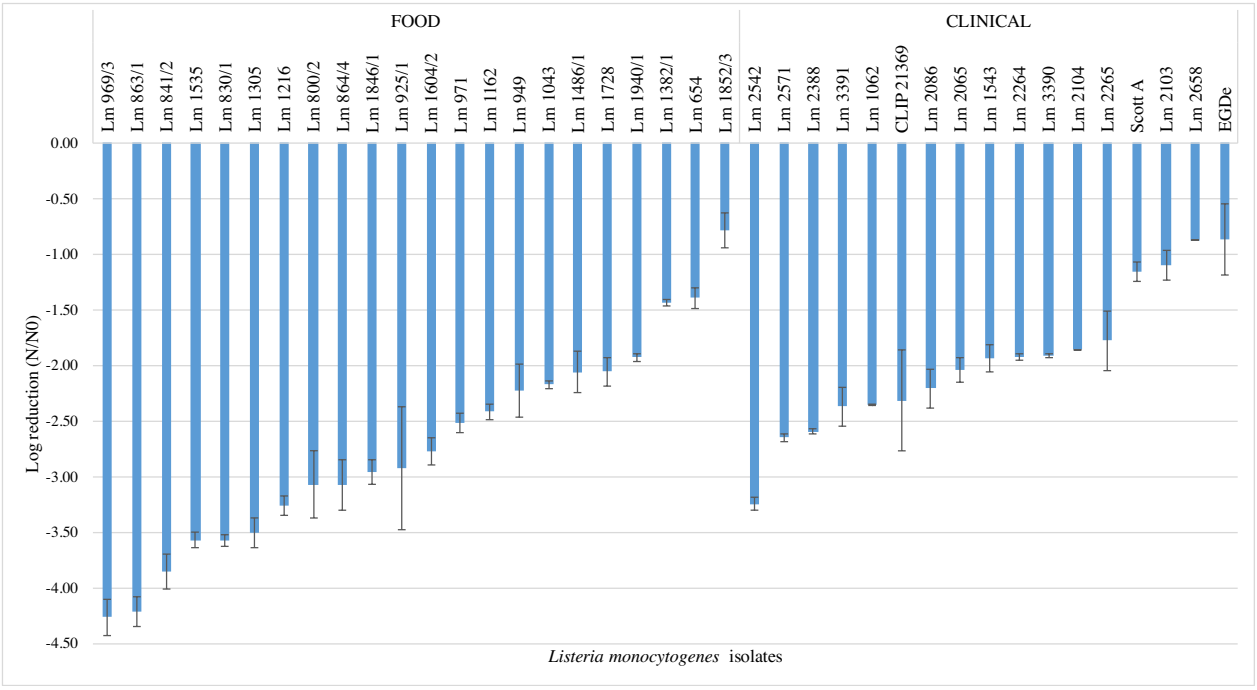

Figure 3.2 *Log reduction of food and clinical isolates of L. monocytogenes strains after 30 sec exposure to 1:10 dilution of red wine, rank-ordered according to their susceptibility. Data represent mean of duplicate assays and error bars represent the standard deviation of the mean.*

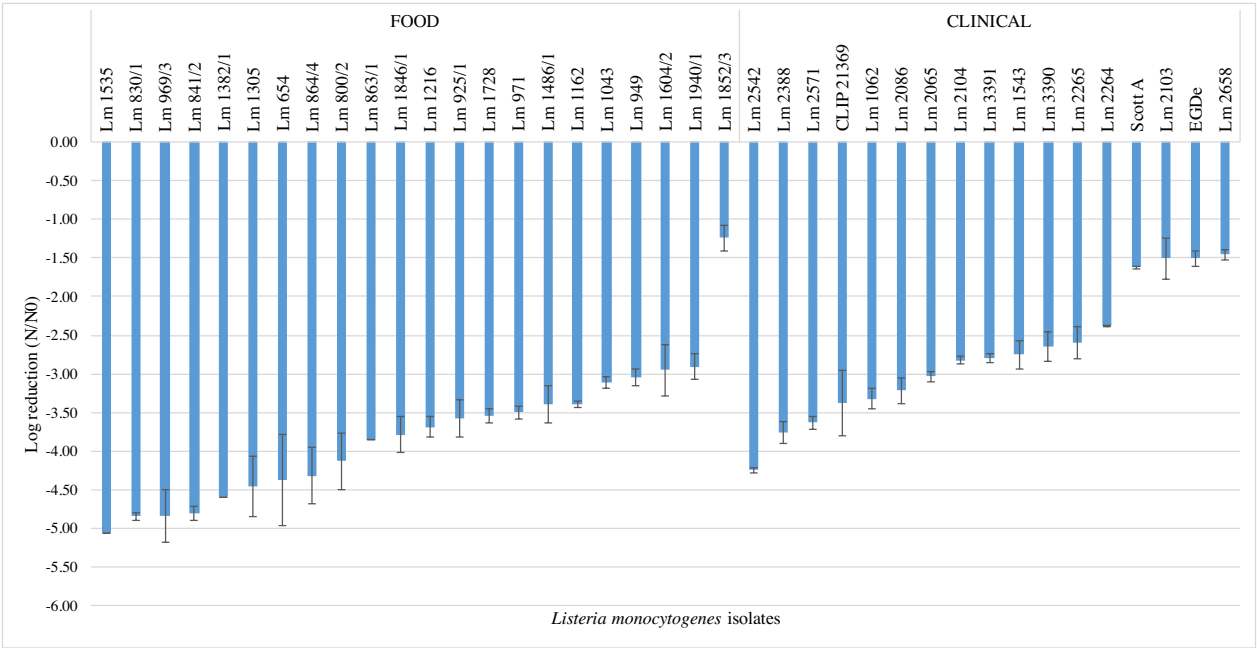

Figure 3.3 *Log reduction of food and clinical isolates of L. monocytogenes strains after 60 sec exposure to 1:10 dilution of red wine, rank-ordered according to their susceptibility. Data represent mean of duplicate assays and error bars represent the standard deviation of the mean.*

Five isolates (all from food origin) presented a reduction of > 3 log cycles immediately after 15 sec of exposure time, while only one clinical isolate showed the same degree of decline, but at 60 sec of exposure time. The food isolate Lm 1852 (serogroup IIb) was the most resistant exhibiting a log reduction of only -1.5 at the end of the experimental period, followed by the clinical isolates Lm 2658 (serogroup IIb), Lm 2103 (serogroup IIa), and reference strain Scott A (serotype 4b) with log reduction values of -1.7, -2.0, and -2.2, respectively. The remain isolates presented reduction values of > 3.0 log cycles at the end of the experimental period.

In the selection of isolates for this study, we have included three clinical (Lm 2264, Lm 2265, and Lm 2671) and three food (Lm 830/1, Lm 841/2, and Lm 864/4) isolates that are grouped into two genotypes (table 1). The clinical isolates that share the same PFGE type 53 were isolated from three different patients in July 2008 and May 2010, in Portuguese hospitals located in the Centre and in the Lisboa e Vale do Tejo regions. The food isolates that share the PFGE type 133 were isolated from ready-to-eat foods produced in the same processing plant in August and October 2003 (Magalhães *et al*., 2015). Isolates of the same PFGE type performed similarly, not presenting significant differences in their susceptibility to the wine ($p < 0.05$).

An overall ANOVA analysis indicated that mean values of log reduction of clinical and food isolates were statistically different ($p < 0.05$) at all sampling times (figure 3.4); food isolates were found to be more susceptible to wine, presenting higher log reduction means (more than one log-cycle reduction) than the clinical isolates. No statistical differences were found ($p > 0.05$) among serogroups IVb, IIb, and IIa, while serogroup IIc isolates were significantly more susceptible to wine ($p < 0.05$); however only two isolates of the latter were analyzed, hence further studies with more IIc isolates should be performed to validate this result.

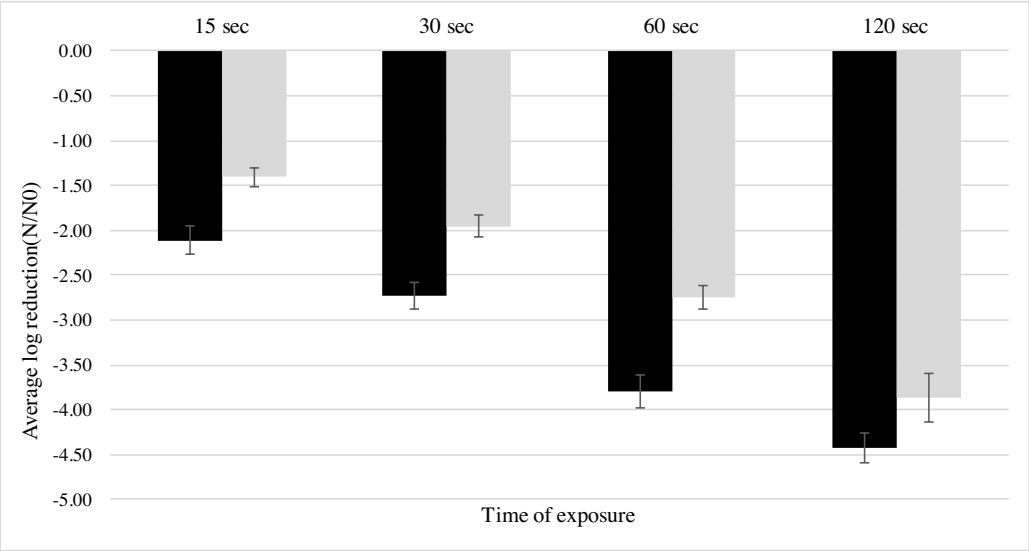

Figure 3.4 Mean log reduction of *L. monocytogenes* isolates of food and clinical origin exposed to red wine (1:10 dilution) at each sampling time *Data represent mean of duplicate assays and error bars represent the standard deviation of the mean*. (■) Food isolates; (■) Clinical Isolates.

Antimicrobial effects of certain beverages such as coffee, tea, beer and wine have been widely reported in the literature (L'Anthoën & Ingledew, 1996; Almeida *et al*., 2006; Medina *et al*., 2007; Carneiro *et al*., 2008). Among them, wine is well known for its antibacterial properties (Møretrø & Daeschel, 2006), and despite its activity against foodborne pathogens like *Campylobacter* spp*., Escherichia coli*, *Salmonella* spp., or *Staphylococcus aureus* (Sheth *et al*., 1988; Just & Daeschel, 2003; Rodríguez Vaquero *et al*., 2007; Carneiro *et al*., 2008) has been extensively investigated, few studies have explored the effect of wine on the survival of *L. monocytogenes*. To measure the effects of wine-related stress exposure on *L. monocytogenes*, we compared the survival response of 39 food and clinical isolates. Our results indicate that, under the tested conditions, red wine had a strong antilisterial activity, and that there is intra-strain variability in the resistance of this pathogen to wine, the human clinical isolates being significantly more resistant than the isolates obtained from food products. Komora *et al*. (2016), using the same isolates of the present study, also reported a higher resistance of clinical isolates to lactic acid and to osmotic stress. As these strains have been characterized in terms of D-value at 58 ºC, antibiotic resistance, and resistance to lactic acid, we evaluated a possible association between resistance to different stresses, however no correlation was found (data not shown).

Fontoura (2012), in a study with eight clinical and eight food *L. monocytogenes* isolates, also found variability on the behavior of this pathogen to diluted red wine, and that clinical isolates were significantly more resistant when submitted to a 1:100 dilution of red wine. However, contradictory to our results, when the three more resistant strains from each origin were challenged in red wine diluted to 1:10 (at 20 ºC), the differences in bacterial cell inactivation between the origins was not significant. Other studies have reported higher resistance of clinical isolates to different environmental stress conditions when compared to food isolates. Variation on isolates of *L. monocytogenes* response to environmental stresses such as temperature, salt, pH, or sanitizers has also been reported (Aryani *et al*., 2015; Magalhães *et al*., 2015; Cunha *et al*., 2016)

Although several studies have explored the major wine components that may play a critical role in bacterial inactivation (e.g. pH, ethanol, organic acids, phenols, etc.) a consensus as not been achieved most likely because wine is a complex solution that incorporates multiple elements; hence its antimicrobial activity is rather due to the synergistic interaction of several parts than due to a single factor (Boban *et al*., 2010). Nevertheless, some wine elements have been reported to hold a strong antilisterial activity, such as ethanol (Corral *et al*., 1990) or ethanol in combination with organic acids (Fernandes *et al*., 2007). Also, phenolic compounds such as caffeic acid, rutin, and quercetin have a strong activity (Rodríguez Vaquero *et al*., 2007). Rhodes and co-authors (2006) showed antilisterial activity in red grape juice without ethanol, and demonstrated its association with different polymeric phenolics of grape skin, seeds and juice. Red wine has also been proved to inactivate *L. monocytogenes* cells in 30 min, and this effect was exacerbated when different marinades prepared with wine, oregano leaves, garlic juice, and oregano oil were applied; in this case the inactivation was instantaneous (Friedman *et al*., 2007).

Wine is an acidic environment, primarily due to the presence of tartaric, malic, and lactic acids. The wine low-pH has been pointed as a key impact factor in bacterial inactivation (Waite & Daeschel, 2007). However, in a study by Boban and co-workers (2010) that evaluated the effect of different elements of the wine against *S. enterica* and *E. coli* found that, used in separate, pH and ethanol presented only a minor antibacterial activity, while in combination with other components a synergistic effect was observed; intact wine was the most effective against these pathogens. Just and Daeschel (2003) when comparing wine and grape juice demonstrated a higher antibacterial effect of wine against the same pathogens, even when both beverages presented the same level of acidity. *L. monocytogenes* is able to tolerate low-pH environments, a feature that is crucial for its survival either in food-associated

environments, as in the infection process during passage through the stomach and within the macrophage phagosome. Dykes and Moorhead (2000) found that clinical isolates were less susceptible to acidic stress (pH 2.5) comparing to isolates from meat. Ramalheira *et al*. (2010) and Barbosa *et al*. (2012) concluded that clinical isolates were more resistant than food isolates recovered from various food products during passage through simulated gastro intestinal tract. Oppositely, Cunha *et al*. (2016) evaluated the ability of 33 isolates from food (18) and clinical (15) origin to survive the gastrointestinal conditions and extreme pH values (1.5 – 12) and found no differences in survival among isolates of different origins.

Our understanding of inter-strain variation in *L. monocytogenes* phenotypic response to different stress conditions is still limited, whether the observed differences between isolates reflect specificities related to their ecology (e.g. adaptation to human, animal, food-associated, and natural environments), or entirely to specific genetic qualities, such as lineage or serotype, is still unclear. For example, a number of studies point out that lineage I isolates (predominantly serotype 4b), are overrepresented among isolates from human listeriosis cases, even though some outbreaks have been caused by lineage I serotype 1/2b and lineage II serotype 1/2a isolates, whereas lineage II isolates appear to be overrepresented among food isolates and may be better adapted to a saprotrophic and environmental life style (Nightingale *et al*., 2005; Orsi *et al*., 2011). Comparative studies on the phenotypic behavior of *L. monocytogenes* isolates representative of different origins, and genetic characteristics, are therefore valuable to gather more data to uncover additional diversity and contribute to our understanding on this pathogen ecology. Our results also underline the importance of using a high number of isolates in this type of studies, as it has become clear that using a low number of isolates or reference strains, may provide biased results or that do not fully reflect the entire spectrum of its features.

3.2 Impact of wine on the invasion capacity of *Listeria monocytogenes* into the human intestinal epithelial Caco-2 cells

To evaluate the possible effect of wine on *L. monocytogenes* virulence, we investigated the ability of four selected isolates to invade the human epithelial Caco-2 cells after exposure to wine. From each origin one resistant and one susceptible isolate were selected, namely: clinical isolates Lm 2658 (IIb, resistant) and Lm 2542 (IVb, susceptible); and food isolates Lm 1852/3 (IIb, resistant) and Lm 969/3 (IIb, susceptible). The invasion

efficiency of resistant and susceptible *L. monocytogenes* exposed to diluted wine (1:10) during 15 sec is plotted in figures 3.5 and 3.6, respectively.

The invasion efficiency of the resistant isolates Lm 2658 and Lm 1852/3 after wine exposure was not statistically different of that observed for their respective controls ($p > 0.05$). Oppositely, the clinical isolate Lm 2542 demonstrated enhanced ability to invade the Caco-2 cells after exposed to the wine in comparison to the unexposed control ($p < 0.05$). This strain was isolated from a listeriosis outbreak in Portugal related with contaminated cheese (Magalhães *et al*., 2015). The food isolate Lm 969/3 unexposed to wine showed a low invasion efficiency. It has been demonstrated that attenuated invasion phenotypes in Caco-2 cells are frequently associated with premature stop codons (PMSC) in *inlA*, which encodes the surface protein InlA, that, as detailed previously in the Introduction section, is a key element for the initial bacterium attachment and invasion of intestinal epithelial cells through interaction with the cell host receptor E-cadherin. Strains with PMSC in *inlA* produce a truncated form of InlA that is secreted rather than anchored to the bacterial cell wall (Van Stelten & Nightingale, 2008). Other factors, such as reduced motility and nonsense mutations in *prfA* gene, that regulates the expression of a set of virulence genes have also been previously associated with impairment in invasion ability in Caco-2 cells (Roche *et al*., 2005; Handa-Miya *et al*., 2007; Roberts *et al*., 2009; Ferreira *et al*., 2011). As this is a poor invasive strain no comparison can be made between exposed and unexposed cells, because the initial bacterial numbers used to inoculate the Caco-2 cells monolayers were already low, in the end of the invasion assay the samples collected for enumeration were below the detection limit of the enumeration technique.

Exposure to stress conditions and food-associated environments may affect virulence-related characteristics in *L. monocytogenes*. For example, Garner *et al*. (2006) found that *L. monocytogenes* became more invasive when subjected to high pH, organic acids, and salt.

The effects of wine on the virulence of *L. monocytogenes* are almost certainly complex and might be related to the expression of virulence genes when this pathogen is under stress. For instance, the alternative sigma factor σ^B (encoded by *sigB*) have been identified as regulating *L. monocytogenes* response to several environmental stresses response and virulence gene expression (Kim *et al*., 2004), and also plays a role in infection of human intestinal Caco-2 cells by regulating transcription of InlA (Garner *et al*., 2006). Further studies including more isolates are needed to confirm these results, and to evaluate the expression of virulence genes relevant for this outcome.

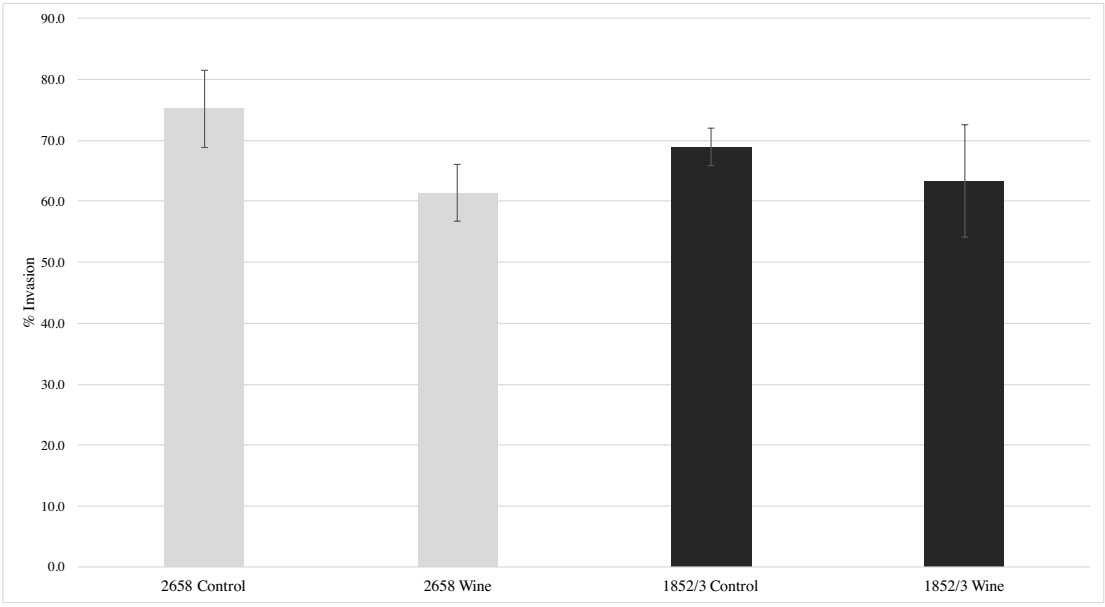

Figure 3.5 *Invasion efficiency of resistant L. monocytogenes clinical (Lm 2658) and food (Lm1852/3) isolates in Caco-2 cells after exposure to wine (1:10 dilution) and their unexposed controls. Data represent the mean of three independent experiment and error bars represent the standard deviation of the mean.*

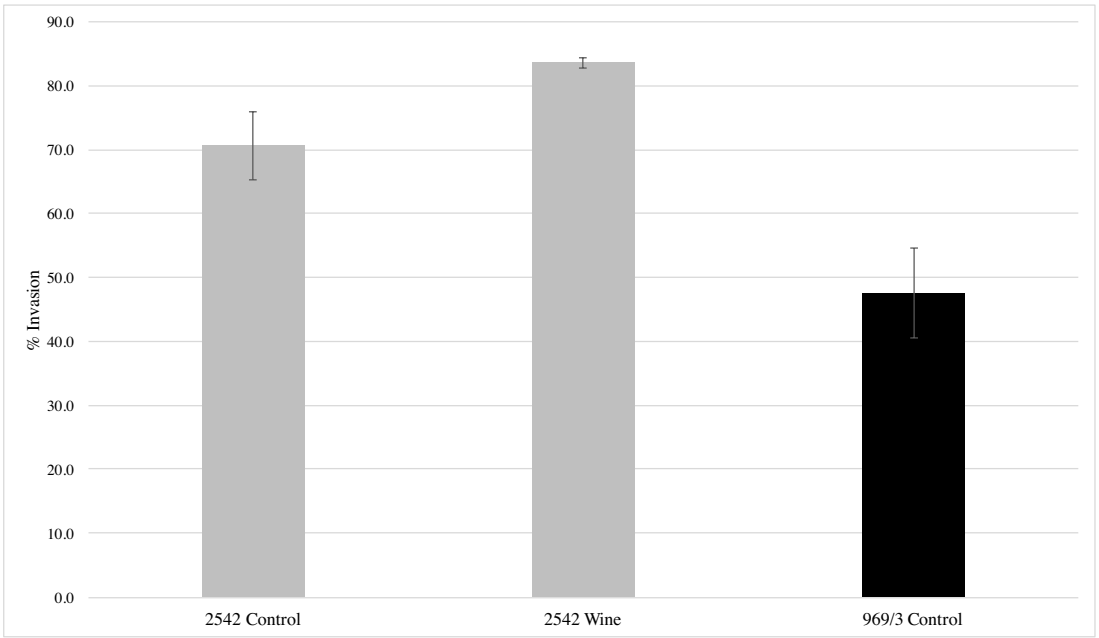

Figure 3.6 *Invasion efficiency of susceptible L. monocytogenes clinical (Lm 2658) and food (Lm1852/3) isolates in Caco-2 cells after exposure to wine (1:10 dilution) and their unexposed controls. Data represent the mean of three independent experiment and error bars represent the standard deviation of the mean. Isolate Lm 969/3 presented naturally an impaired invasion ability and after wine exposure its invasion efficiency was null.*

4 Conclusions

This research aimed to evaluate the effect of red wine on the survival of *L. monocytogenes* isolates from food and clinical origin, and to assess its impact on the pathogen ability to invade human intestinal epithelial cells *in vitro*. A 1:10 solution of red wine demonstrated a strong antilisterial activity, yielding a 4.5 log reduction for the majority of the isolates in only 2 minutes. A high phenotypic variation was observed among isolates, but overall, clinical isolates were significantly less susceptible to wine when compared with food isolates. Exposure to wine enhanced the ability of one clinical strain, associated with a listeriosis outbreak occurred in Portugal, to invade Caco-2 cells. This effect was not observed in two strains demonstrating higher resistance to red wine. A small number of previous studies have sought to examine the effect of wine on *L.* monocytogenes, and this is, as far as we know, the first study using a considerable number of strains (n=39) from different origins and diverse phenotypic and genetic characteristics; furthermore, this is the first study evaluating the influence of red wine on virulence traits of a foodborne pathogen.

Although the extrapolation of the results obtained here to predict the behaviour of *L. monocytogenes* when contaminated foods are in contact with wine should be done in a cautious manner, this study gives evidence that red wine can be seen as a significant barrier to *L. monocytogenes* survival. Our data also indicates that the exposure to wine may influence the invasiveness ability of some strains *L. monocytogenes* into human intestinal cells. Further studies including more isolates should be performed to substantiate this finding and uncover mechanisms involved in this response.

5 Future work

Some following suggestions for future work are:

1. To evaluate the inactivation effect of red wine in *L. monocytogenes* using a wider set of strains, including more setotype 1/2c isolates;
2. To investigate the antilisterial activity of red wine on different food matrices and to evaluate the possible protective effect of food components;
3. To validate the ability of red wine to enhance invasion Caco-2 cells, and other cell lines, efficiency using more strains susceptible to wine, and compare with resistant strains;
4. To explore the mechanisms underlying enhanced invasiveness in outbreak strain Lm 2542, and the expression of virulence genes relevant for this outcome.

6 References

Alessandria, V., Rantsiou, K., Dolci, P., & Cocolin, L. (2010). Molecular methods to assess *Listeria monocytogenes* route of contamination in a dairy processing plant. *International journal of food microbiology*, *141*, S156-S162.

Allerberger, F., & Wagner, M. (2010). Listeriosis: a resurgent foodborne infection. *Clinical Microbiology and Infection*, *16*(1), 16-23.

Almeida, A. A. P., Farah, A., Silva, D. A., Nunan, E. A., & Glória, M. B. A. (2006). Antibacterial activity of coffee extracts and selected coffee chemical compounds against enterobacteria. *Journal of Agricultural and Food Chemistry*, *54*(23), 8738-8743.

Aryani, D. C., Den Besten, H. M. W., Hazeleger, W. C., & Zwietering, M. H. (2015). Quantifying variability on thermal resistance of *Listeria monocytogenes*. *International journal of food microbiology*, *193*, 130-138.

Barbosa, J., Borges, S., Magalhães, R., Ferreira, V., Santos, I., Silva, J., ... & Teixeira, P. (2012). Behaviour of *Listeria monocytogenes* isolates through gastro-intestinal tract passage simulation, before and after two sub-lethal stresses. *Food microbiology*, *30*(1), 24-28.

Barker, C., & Park, S. F. (2001). Sensitization of *Listeria monocytogenes* to low pH, organic acids, and osmotic stress by ethanol. *Applied and environmental microbiology*, *67*(4), 1594-1600.

Bennett, L. (2015). *Listeria monocytogenes*. In *Mandell, Douglas, and Bennett's principles and practice of infectious diseases*. 8th ed, (J. Bennett, R. Dolin & M. Blaser, eds.), Philadelphia: Saunders, 2383–2388.

Boban, N., Tonkic, M., Budimir, D., Modun, D., Sutlovic, D., Punda-Polic, V., & Boban, M. (2010). Antimicrobial effects of wine: separating the role of polyphenols, pH, ethanol, and other wine components. *Journal of food science*, *75*(5), M322-M326.

Bortolussi, R. (2008). Listeriosis: a primer. *Canadian Medical Association Journal*, *179*(8), 795-797.

Bover, S. & Garriga, M. (2014). Condiciones que determinan el crecimiento y la supervivencia de *Listeria monocytogenes* en alimentos listos para el consumo. *IRTA (Institut de recerca y tecnologia agroalimentàries)*, 81.

Carneiro, A., Couto, J. A., Mena, C., Queiroz, J., & Hogg, T. (2008). Activity of wine against *Campylobacter jejuni*. *Food Control*, *19*(8), 800-805.

Corral, F. D., Buchanan, R. L., Bencivengo, M. M., & Cooke, P. H. (1990). Quantitative comparison of selected virulence associated characteristics in food and clinical isolates of *Listeria*. *Journal of Food Protection*, *53*(12), 1003-1009.

Cueva, C., Moreno-Arribas, M. V., Martín-Álvarez, P. J., Bills, G., Vicente, M. F., Basilio, A., López Rivas, C., Requena, T., Rodríguez J. M., & Bartolomé, B. (2010). Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. *Research in microbiology*, *161*(5), 372-382.

Cunha, S., Komora, N., Magalhães, R., Almeida, G., Ferreira, V., & Teixeira, P. (2016). Characterization of clinical and food *Listeria monocytogenes* isolates with different antibiotic resistance patterns through simulated gastrointestinal tract conditions and environmental stresses. *Microbial Risk Analysis*, *1*, 40-46.

Doyle, M.E. (2001). Virulence Characteristics of *Listeria Monocytogenes*. *Food Research Institute*, *October*, 1-21.

Dykes, G. A., & Moorhead, S. M. (2000). Survival of osmotic and acid stress by *Listeria monocytogenes* strains of clinical or meat origin. *International journal of food microbiology*, *56*(2), 161-166.

European Food Safety Authorty (2015). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. *EFSA Journal*, 13(12), 4329.

Euzéby, J. (2015). List of prokaryotic names with standing in nomenclature. Available at: http://www.bacterio.net/listeria.html [Accessed: February 27, 2016].

Farber, J. M., & Peterkin, P. I. (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiological reviews*, *55*(3), 476-511.

Fernandes, J., Gomes, F., Couto, J. A., & Hogg, T. (2007). The antimicrobial effect of wine on *Listeria innocua* in a model stomach system. *Food Control*, *18*(12), 1477-1483.

Ferreira, V., Barbosa, J., Stasiewicz, M., Vongkamjan, K., Switt, A. M., Hogg, T., Gibbs, P., Teixeira, P., & Wiedmann, M. (2011). Diverse geno-and phenotypes of persistent *Listeria monocytogenes* isolates from fermented meat sausage production facilities in Portugal. *Applied and environmental microbiology*, *77*(8), 2701-2715.

Fontoura, G.M.F. (2012). Actividade do vinho contra *Listeria monocytogenes* - comparação de isolados alimentares com clínicos [Master's thesis]. Universidade Católica Portuguesa: Porto.

Frankel, E. N., Waterhouse, A. L., & Teissedre, P. L. (1995). Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting

oxidation of human low-density lipoproteins. *Journal of Agricultural and Food chemistry*, *43*(4), 890-894.

Friedman, M. (2014). Antibacterial, antiviral, and antifungal properties of wines and winery byproducts in relation to their flavonoid content. *Journal of agricultural and food chemistry*, *62*(26), 6025-6042.

Friedman, M., Henika, P. R., Levin, C. E., & Mandrell, R. E. (2007). Recipes for antimicrobial wine marinades against *Bacillus cereus*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica*. *Journal of food science*, *72*(6), M207-M213.

Garner, M. R., Njaa, B. L., Wiedmann, M., & Boor, K. J. (2006). Sigma B contributes to *Listeria monocytogenes* gastrointestinal infection but not to systemic spread in the guinea pig infection model. *Infection and immunity*, *74*(2), 876-886.

Garner, M. R., James, K. E., Callahan, M. C., Wiedmann, M., & Boor, K. J. (2006). Exposure to salt and organic acids increases the ability of *Listeria monocytogenes* to invade Caco-2 cells but decreases its ability to survive gastric stress. *Applied and Environmental Microbiology*, *72*(8), 5384-5395.

Handa-Miya, S., Kimura, B., Takahashi, H., Sato, M., Ishikawa, T., Igarashi, K., & Fujii, T. (2007). Nonsense-mutated inlA and prfA not widely distributed in *Listeria monocytogenes* isolates from ready-to-eat seafood products in Japan. *International journal of food microbiology*, *117*(3), 312-318.

Jackson, R.S. (2014). *Wine Science: Principles and Applications* 4th ed., USA: Academic Press, 347 -370; 894-899.

Just, J. R., & Daeschel, M. A. (2003). Antimicrobial effects of wine on *Escherichia coli* O157:H7 and *Salmonella typhimurium* in a model stomach system. *Journal of food science*, *68*(1), 285-290.

Király-Véghely, Z., Móricz, Á. M., Ott, P. G., Kátay, G., Belai, I., & Tyihák, E. (2009). Comparison of components from red and white wines for antimicrobial activity by biodetection after OPLC separation. *Journal of Liquid Chromatography & Related Technologies*, *32*(9), 1259-1272.

Komora, N., Braschi, C., Magalhães, R., Ferreira, V., Teixeira, P. 2016 .Impact of antibiotic resistance on the survival of *Listeria monocytogenes* exposed to food-associate stresses. *International Journal of Food Microbiology. Submitted for publication*

L'Anthoën, N.C.L., & Ingledew, W.M. (1996). Heat resistance of bacteria in alcohol-free beer. *Journal of. American. Society of. Brewing. Chemists*, 54(1),32–36.

Ledermann, W. (2007). *Una historia personal de las bacterias*. Santiago: RIL editores, 135–144.

Liu, C., Chen, R., & Su, Y. C. (2006). Bactericidal effects of wine on *Vibrio parahaemolyticus* in oysters. *Journal of Food Protection*, *69*(8), 1823-1828.

Ludwig, W., Schleifer, K.-H. & Whitman, W.B. (2001). Family III. *Listeriaceae*. In *Bergey's manual of systematic bacteriology*. *Volume three: The Firmicutes*, 2th ed, (P. De Vos, G. M. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K.-H. Schleifer & W. B. Whitman, eds.), USA: Springer, 244-261.

Magalhães, R., Almeida, G., Ferreira, V., Santos, I., Silva, J., Mendes, M. M., Pita, J., Mâncio, I., Sousa, M. M., Farber, J., Pagotto, F., & Teixeira, P. (2015). Cheese-related listeriosis outbreak, Portugal, March 2009 to February 2012. *Euro surveillance*, *20*(17), 7), 21104.

McDonnell, G., & Russell, A. D. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clinical microbiology reviews*, *12*(1), 147-179.

Medina, E., Romero, C., Brenes, M., & de Castro, A. (2007). Antimicrobial activity of olive oil, vinegar, and various beverages against foodborne pathogens. *Journal of Food Protection*, *70*(5), 1194-1199.

Monagas, M., & Bartolomé, B. (2009) *Anthocyanins and Anthocyanin-Derived Compounds* In *Wine Chemistry and Biochemistry,* (M.V. Moreno-Arribas & M.C. Polo, eds.), New York: Springer, 439-442.

Møretrø, T., & Daeschel, M. A. (2004). Wine is bactericidal to foodborne pathogens. *Journal of Food Science*, *69*(9), M251-M257.

Muñoz-González, I., Thurnheer, T., Bartolomé, B., & Moreno-Arribas, M. V. (2014). Red wine and oenological extracts display antimicrobial effects in an oral bacteria biofilm model. *Journal of agricultural and food chemistry*, *62*(20), 4731-4737.

Ng, W. K., & Koh, C. B. (2016). The utilization and mode of action of organic acids in the feeds of cultured aquatic animals. *Reviews in Aquaculture*, *0*, 1-27.

Nightingale, K. K., Windham, K., Martin, K. E., Yeung, M., & Wiedmann, M. (2005). Select *Listeria monocytogenes* subtypes commonly found in foods carry distinct nonsense mutations in inlA, leading to expression of truncated and secreted internalin A, and are associated with a reduced invasion phenotype for human intestinal epithelial cells. *Applied and environmental microbiology*, *71*(12), 8764-8772.

Orsi, R. H., den Bakker, H. C., & Wiedmann, M. (2011). *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. *International Journal of*

Medical Microbiology, *301*(2), 79-96.

Paulo, L., Oleastro, M., Gallardo, E., Queiroz, J. A., & Domingues, F. (2011). Anti-*Helicobacter pylori* and urease inhibitory activities of resveratrol and red wine. *Food Research International*, *44*(4), 964-969.

Phan-Thanh, L., & Montagne, A. (1998). Physiological and biochemical aspects of the acid survival of *Listeria monocytogenes*. *The Journal of general and applied microbiology*, *44*(3), 183-191.

Ramalheira, R., Almeida, M., Azeredo, J., Brandao, T. R., Almeida, G., Silva, J., & Teixeira, P. (2010). Survival of clinical and food isolates of *Listeria monocytogenes* through simulated gastrointestinal tract conditions. *Foodborne pathogens and disease*, *7*(2), 121-128.

Rentzsch, M., Wilkens, A., & Winterhalter, P. (2009) *Non-flavonoid Phenolic Compounds*. In *Wine Chemistry and Biochemistry,* (M.V. Moreno-Arribas & M.C. Polo, eds.), New York: Springer, 509-512.

Rhodes, P. L., Mitchell, J. W., Wilson, M. W., & Melton, L. D. (2006). Antilisterial activity of grape juice and grape extracts derived from *Vitis vinifera* variety Ribier. *International journal of food microbiology*, *107*(3), 281-286.

Roberts, A. J., Williams, S. K., Wiedmann, M., & Nightingale, K. K. (2009). Some *Listeria monocytogenes* outbreak strains demonstrate significantly reduced invasion, inlA transcript levels, and swarming motility in vitro. *Applied and environmental microbiology*, *75*(17), 5647-5658.

Roche, S. M., Gracieux, P., Milohanic, E., Albert, I., Virlogeux-Payant, I., Temoin, S., Grépinet, O., Kerouanton, A., Jacquet, C., Cossart, P., & Velge, P. (2005). Investigation of specific substitutions in virulence genes characterizing phenotypic groups of low-virulence field strains of *Listeria monocytogenes*. *Applied and environmental microbiology*, *71*(10), 6039-6048.

Rodríguez Vaquero, M. J., Alberto, M. R., & de Nadra, M. C. M. (2007). Influence of phenolic compounds from wines on the growth of *Listeria monocytogenes*. *Food Control*, *18*(5), 587-593.

Rodríguez Vaquero, M. J., Alberto, M. R., & de Nadra, M. C. M (2007). Antibacterial effect of phenolic compounds from different wines. *Food Control*, *18*(2), 93-101.

Sheth, N. K., Wisniewski, T. R., & Franson, T. R. (1988). Survival of enteric pathogens in common beverages: An *in vitro study*. *American Journal of Gastroenterology*, *83*(6), 658-660.

Soleas, G. J., Diamandis, E. P., & Goldberg, D. M. (1997). Wine as a biological fluid: history, production, and role in disease prevention. *Journal of clinical laboratory analysis*, *11*(5), 287-313.

Sugita-Konishi, Y., Hara-Kudo, Y., Iwamoto, T., & KoNDo, K. (2001). Wine has activity against entero-pathogenic bacteria *in vitro* but not *in vivo*. *Bioscience, biotechnology, and biochemistry*, *65*(4), 954-957.

Swaminathan, B., Cabanes, D., Zhang, W., & Cossart, P. (2007). *Listeria monocytogenes*. In *Food Microbiology: Fundamentals and Frontiers,* 3th ed*,* (L. & M. P. Doyle & R. Beuchat, eds.), Washington, D. C.: American Society of Microbiology, 457-480.

Takkouche, B., Regueira-Méndez, C., García-Closas, R., Figueiras, A., Gestal-Otero, J. J., & Hernán, M. A. (2002). Intake of wine, beer, and spirits and the risk of clinical common cold. *American Journal of Epidemiology*, *155*(9), 853-858.

Terrier, N., Poncet-Legrand C., & Cheynier, V. (2009) *Flavanols, Flavonols and Dihydroflavonols*. In *Wine Chemistry and Biochemistry,* (M.V. Moreno-Arribas & M.C. Polo, eds.), New York: Springer, 463-465.

Van Stelten, A., & Nightingale, K. K. (2008). Development and implementation of a multiplex single-nucleotide polymorphism genotyping assay for detection of virulence-attenuating mutations in the *Listeria monocytogenes* virulence-associated gene inlA. *Applied and Environmental Microbiology*, *74*(23), 7365-7375.

Vaz, M., Hogg, T., & Couto, J. A. (2012). The antimicrobial effect of wine on *Bacillus cereus* in simulated gastro-intestinal conditions. *Food Control*, *28*(2), 230-236.

Vázquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., & Kreft, J. (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clinical microbiology reviews*, *14*(3), 584-640.

Waite, J. G., & Daeschel, M. A. (2007). Contribution of wine components to inactivation of food-borne pathogens. *Journal of food science*, *72*(7), M286-M291.

Walker, S. J., Archer, P., & Banks, J. G. (1990). Growth of *Listeria monocytogenes* at refrigeration temperatures. *Journal of Applied Bacteriology*, *68*(2), 157-162.

Zorn, B.G. & Suárez, R., 2009. *Listeria* y Listeriosis. *Profesión veterinaria*, *Abr-Jul*(71), 58–67.