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UNIVERSIDADE CATÓLICA PORTUGUESA
ESCOLA SUPERIOR DE BIOTECNOLOGIA

Title: ***Listeria monocytogenes*****, Cows Raw Milk, Mastitis and Food Safety**

Thesis presented to Escola Superior de Biotecnologia of the Universidade Católica Portuguesa to achieve the Master of Science level in Food Science and Technology

By

Jorge Manuel Pinto Ferreira

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Abstract

This thesis intends to initially make an overview of the food safety area, and then a review of the main topics about *L. monocytogenes*, particularly its relationship with milk and the dairy industry, and report the laboratory work done in the scope of a Master of Science (MS) project. The principal aim of the work was the evaluation of the occurrence of *L. monocytogenes* in cows raw milk, in the Northern Portuguese Coast region. The detection (with VIDAS methodology) and enumeration (direct and according with the Most Probable Number (MPN) technique) of *L. monocytogenes* was performed in a total of 166 raw milk samples (45 from healthy cows, 58 from cows presenting sub-clinical mastitis, 27 from cows presenting clinical mastitis, and 36 were bulk tank samples), from 39 different dairy farms, from 13 different localities. *L. monocytogenes* was detected in two of these samples: one from a bulk tank, and the other from a clinical mastitis, both from the same dairy farm. From this dairy, environmental samples, as well as a sample of silage, *unifeed*, cattle manure and water were also analysed, for detection and enumeration of *L. monocytogenes*. To our knowledge, this was the first time *L. monocytogenes* was isolated from a clinical mastitis cow milk in Portugal. Twenty two *L. monocytogenes* isolates (from the refered milk samples) were identified, and further characterized by multiplex Polymerase Chain Reaction (PCR), antibiotic susceptibility and resistance to arsenic, cadmium and tetracycline. These subtyping techniques had results similar for all the isolates (with the exception of gentamicin Minimum Inhibitory Concentration (MIC)) suggesting that all the isolates belong to the same clone, particularly adapted to the environment of that farm, and that the origin of bulk tank contamination, was an infected cow, excreting *L. monocytogenes* through the udder. Additional studies using more discriminatory techniques such as pulse field gel electrophoresis (PFGE), however, are needed in order to confirm this hypothesis.

Resumo

Esta tese pretende, inicialmente, abordar genericamente a área da segurança alimentar, fazer depois uma revisão sobre os principais tópicos acerca de *L. monocytogenes* (particularmente a sua relação com o leite e com a indústria leiteira) e, por fim, relatar o trabalho laboratorial que foi feito integrado no meu projecto de Mestrado. O nosso principal objectivo era avaliar a ocorrência deste agente em leite cru de vacas leiteiras, na região norte, litoral, de Portugal. A detecção (realizada através da metodologia VIDAS) e a enumeração (directa e de acordo com a técnica do Número Mais Provável (NMP)) de *L. monocytogenes* foi investigada num total de 166 amostras de leite cru de vaca (45 amostras de vacas saudáveis, 58 de vacas com mamite sub-clinica, 27 de vacas com mamites clínicas e 36 tanques de leite), de 39 explorações, de 13 localidades diferentes. *L. monocytogenes* foi detectada em 2 destas amostras: 1 de um tanque e uma de uma mamite clínica, ambas do mesmo produtor. Tendo por base a pesquisa bibliográfica efectuada, esta foi a primeira vez que *L. monocytogenes* foi isolada de leite mastitico de vaca, em Portugal. Nesta exploração, foram ainda recolhidas e analisadas (para detecção e enumeração de *L. monocytogenes)* amostras ambientais, assim como uma amostra de silagem, *unifeed*, fezes e água. Foram identificados (das amostras de leite referidas) 22 isolados de *L. monocytogenes,* que foram caracterizados por uma técnica de multiplex PCR, testes de susceptibilidade antibiótica e resistência a arsénio, cádmio e tetraciclina. Tendo em conta que os resultados destes testes foram muito semelhantes para todos os isolados (com a excepção da sensibilidade a gentamicina), isto parece sugerir que todos os isolados pertencem ao mesmo clone, particularmente adaptado ao ambiente daquela exploração, e que a origem da contaminação do tanque pode ter sido uma vaca, a excretar *L. monocytogenes* através do úbere. São, no entanto, necessários estudos adicionais, com técnicas mais discriminatórias (ex. Electroforese em Campo Pulsado (*PFGE*)), para confirmar estas hipóteses.

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Contents

Abbreviations

AIDS – Acquired imuno deficiency syndrome
ALOA - Agar Listeria Ottavani & Agosti
ARDS- Adult respiratory distress syndrome
ATCC – American Type Culture Collection
BMP – Best Management Practices
BTSCC – Bulk tank somatic cell count
cAMP- cyclic adenosine monophosphate
CSF – Cerebral Spinal Fluid
cfu- colony forming units
CIP – Collection de l´ Institute Pasteur
CMT – Californian Mastitis Test
CNS **-** Central nervous system
DIC - Disseminated Intravascular Coagulation
EFSA- European Food Standard Agency
e.g.- example
ELFA – Enzyme-linked Fluorescent Immunoassay
EM- Electronic Microscopy
ERS – Economic Research Service
EU – European Union
FDA- Food and Drug Administration
HACCP -Hazard Analysis Critical Control Point
HPA – Health Protection Agency
i.e. – in essence
G+C – Guanyne+Cytosine
GI - Gastrointestinal
LMO – *Listeria monocytogenes*
MIC – Minimum Inhibitory Concentration
MPN – Most Probable Number
MRI - Magnetic Resonance Imaging
MS – Master of Science
NCCLS – National Committee for Clinical Laboratory Standards
PALCAM - Polymixin Acriflavine LiCl Ceftazidime Aesculin Mannitol
PCR- Polymerase Chain Reaction
PFGE – Pulse Field Gel Electrophoresis
RAPD –Random Amplified Polymorphic DNA
SCC- Somatic Cell Count
SDS – Sodium Dodecyl Sulphate
SPR – Solid Phase Receptacle
st - sterile
SVA – Serviços Veterinários Associados
TSAYE – Tryptone-soy agar yeast extract
TSBYE - Tryptone-soy broth yeast extract
U- Units
UP – Ultra Pure
USA – United States of America
USDA- United States Department of Agriculture
Vet.- veterinarians

I. PREFACE

As far as I can remember, I have always wanted to become a veterinarian, but when I started the veterinary medicine course, I was not sure if I would like to work with small or food animals, or in any other related area. On the 4th year of Veterinary school, it seemed to me that working with food animals would better fit my personality. So I decided to do my internship training in this area and have been working as a dairy cattle clinician for the last 5 years.

After an initial period of euphoria when all I wanted was to do clinical work, I started to feel the need to deepen my knowledge about other subjects related to my work, namely Veterinary Public Health and Food Safety. For that reason I did a post-graduate program in Food Safety, which allowed me to learn many new concepts, but also made me realize how much I have still to learn on this field. This reinforced my conviction that pursuing a Master of Science (MS) was the next logical step, as I also find these kind of studies a great opportunity to join the sometimes too apart academic and business worlds.

Working and studying simultaneously was an enriching and challenging experience that has ultimately strengthened my belief that keeping contact with the field work while developing study/research work, may limit the quality of the work that is done, but is fundamental to achieve results that can be actually put into practice. With this in mind, I started to look for a theme that could be interesting and useful for me as a clinician and also important as a public health issue, and, therefore, chose "*Listeria monocytogenes*, Cows Raw Milk, Mastitis and Food Safety" as my MS dissertation theme…why?

1) It is somehow intriguing that in 25-40% of all mastitic milk samples analysed in the laboratory (blood agar and MacConkey media) there is no growth for bacterial agents. There are several possible explanations for this fact (that will be mentioned further on in this thesis); one of them is that, maybe, some of the possible agents, such as *L. monocytogenes* are not being searched for.

2) *L. monocytogenes* is among the five most common bacterial agents of meningitis and, although uncommon, is also an important cause of abortions. Listeriosis prsents a high mortality rate (25-50%), but data about this agent in Portugal are scarce.

3) Based on developments within the dairy sector as well as at the European Union political level, it can be expected that the application of Hazard Analysis Critical Control Points (HACCP)-compatible programmes on the dairy farms will be conducted in the near future. In order to establish international microbiological criteria for *L. monocytogenes* in foods, it is necessary to know the prevalence of the pathogen at different points in the food processing chain, from "manufacturing" to the consumer.

Having these in mind, a MS project was developed, and the principal aim of the work was the evaluation of the occurrence of *L. monocytogenes* in cows raw milk, in the Northern Portuguese coast region.

II. INTRODUCTION

In this chapter, an introduction to the Food Safety area will be done, with particular attention to the dairy industry related themes. *L. monocytogenes* relation with "milk" will also be reviewed.

We hear much these days about food safety. What's the concern? More than 200 known diseases are transmitted through food by a variety of agents that include bacteria, fungi, viruses, and parasites. According to public health and food safety experts, each year millions of illnesses throughout the world can be traced to foodborne pathogens. The risk of foodborne illness has increased markedly over the last 20 years, with nearly a quarter of the population at higher risk of illness today (Oliver *et al*., 2005). The situation becomes even more problematic because of rapidly changing demographics, with an increasing number of elderly people and immunocompromised individuals who are more susceptible to foodborne pathogens (Notermans & Hoogenboom-Verdegaal, 1992). Consequently, preventing illness and death associated with foodborne pathogens remains a major public health challenge.

Why has the risk of foodborne illness increased? There are several reasons. Much has changed in what we eat and where we eat. A greater variety of foods are consumed, particularly seafood, fresh fuits, and fresh vegetables, and consumers demand these foods year round. To satisfy this demand, more foods are imported from foreign countries. Another factor is that more meals are eaten away from home. As more people become involved in preparing our meals, the chance for foodborne illness increases dramatically (Oliver *et al*., 2005).

On the other hand, consumers are increasingly concerned about the safety of their food and uncertain about food production practices (Ruegg, 2003). Modern communication systems have enhanced consumer awareness of outbreaks occurring throughout the world and have reduced the sense of safety associated with distance. Geographical barriers to the spread of diseases have been reduced by the globalization of food systems and by the frequent movement of people and animals. Consumer confidence in existing food handling and processing systems has been reduced by the occurrence of various problems related with the safety of foods. The emergence of

transmissible spongiform encephalopathies associated with animal products (Brown *et al*., 2001) was probably the most significant.

II. 1 Economic and social losses

It is well-estabilished that foodborne diseases cause significant economic and social losses due to, for example, absenteeism, medical care, investigations, withdrawal of the contaminated products and loss of confidence in products (Leclerc *et al.*, 2002). The economic impact of foodborne diseases on society is, in fact, stagerring. In 1993, the Economic Research Service (ERS) of the USDA (United States Department of Agriculture) indicated that the annual cost of human disease caused by the more common foodborne pathogens ranged from $5.6 to $9.4 billion dollars (Busby & Roberts, 1995). The number of cases of foodborne disease caused by *E. coli* O157:H7 ranged between 8,000 and 16,000 with 400 deaths and a cost between $200 and $600 million dollars. For *Salmonella* species, the number ranged from 696,000 to 3,840,000 with 3,840 deaths and an estimated cost between $600 million to 3.5 billion dollars (Busby & Roberts, 1995). According to the USDA ERS estimates, medical costs, productivity losses and value of premature deaths for diseases caused by five major foodborne bacterial pathogens approach $7 billion per year. Cost estimates for the year 2000 were $1.2 billion for *Campylobacter* (all serotypes), $2.4 billion for *Salmonella* (nontyphoidal), $0.7 billion for *E.coli* O157:H7, $0.3 billion for non O157 Shiga-toxin producing *E. coli*, and $2.3 billion for *L. monocytogenes*. It is therefore evident that reducing foodborne pathogen contamination in the food chain could save both lives and billions of dollars in costs annually (Oliver *et al*., 2005).

II. 2 Dairy industry

The dairy industry has been extremely successful in producing safe and nutritious products. Milk is a highly nutritious food that is ideally suited for growth of pathogenic organisms. Nevertheless, it contains low numbers of bacteria when it is milked under good hygienic conditions from healthy cows (Salo *et al*., 2006). Consumption of raw milk remains a well-identified risk factor for foodborne disease, but pasteurization (and other thermal treatments) has been highly effective in ensuring the safety of dairy products (Headrick *et al*., 1998). Even though dairy products are consumed on a daily

basis, milk, ice cream, and cheese have been identified as the vehicle for less than 1.5% of all foodborne disease outbreaks investigated by the Centers for Disease Control (Bean *et al*., 1996).

Anyway, should the dairy industry be also concerned about food safety? Yes, and there are several good reasons (Oliver *et al.,* 2005) for that:

1) bulk tank milk contains several foodborne pathogens that cause human disease. Healthy dairy cattle are considered a reservoir for several of the most important foodborne human pathogens (Tauxe, 1997). Nontyphoidal *Salmonella* spp., and *C. jejuni* are considered important threats to food safety because of the enormous number of illnesses they cause. *L. monocytogenes* and *E. coli* O157:H7 are considered priority pathogens because of the severity of symptoms associated with infection they cause and because of the number of deaths that occur in infected people. All these pathogens are shed in cattle faeces and can contaminate dairy farms premises including unpasteurized bulk tank milk. For example, between 2-16% of healthy cows excrete *L. monocytogenes* in faeces for months to years (Muraoka *et al*., 2003). In some instances, colonization of the udder can also contribute to contamination of bulk milk supplies. *Salmonella* spp., are an infrequent cause of mastitis in dairy cows but several species of *Salmonella* have been documented to colonize udders and shed at levels of up to 2000 organism/mL (Fontaine *et al*., 1980). *L. monocytogenes* also has been reported to cause mastitis and can be shed in milk (Ruegg, 2003), a subject that will be further detailed in this thesis (section IV.1.1.1). A study that examined more than 500 isolates of milk obtained from coliform mastitis cases was not able to isolate O157:H7 from any of the samples and *E. coli* O157:H7 has not been recognized as a cause of mastitis (Cullor, 1997). *C. jejuni* can also be shed in milk, but faecal contamination of milk is a more likely route of exposure. It should although be emphasied that when regulatory standards for bacterial counts in raw milk are met, pasteurization of milk is highly effective in destroying all of these organisms (Ruegg, 2003). For example, coliform counts should be less than 100 cfu/mL for milk intended to be pasteurized before consumption and less than 10 cfu/mL if raw milk will be consumed (Ruegg, 2003).

2) outbreaks of disease in humans have been traced back to pasteurized milk. In cases involving pasteurized dairy products, errors in the pasteurization process or the

addition of nonpasteurized eggs have frequently been identified as the route of contamination (Ruegg, 2003). The literature shows that *L. monocytogenes* has also been isolated from pasteurized milk, besides raw milk and mastitic milk. Other dairy products associated with listeriosis (table 1) outbreaks include unpasteurized soft cheese, butter, unpasteurized milk and ice-cream (Salo *et al*., 2006).

Table 1: Prevalence of *L. monocytogenes* in different types of dairy products in European countries (*in* Lundén *et al*., 2004).

Product	Prevalence (%) of *Listeria monocytogenes*	Country of origin	Reference
Raw milk	4.4	The Netherlands	Beckers *et al*., 1987
Raw milk	3.6	England & Wales	Greenwood *et al*., 1991
Raw milk	1	Sweden	Waak *et al*., 2002
Soft cheese made of raw milk	65	France	Beckers *et al*., 1987
Soft ripened cheese	8.2	England & Wales	Greenwood *et al*., 1991
Soft unripened cheese	1.1	England & Wales	Greenwood *et al*., 1991
Soft cheese	6	Italy, Germany, Áustria & France	Rudolf & Scherer, 2001
Soft or semi soft cheese	6	Italy, Germany & France	Loncarevic *et al*., 1995
Semi soft cheese	8	Italy, Germany, Áustria & France	Rudolf & Scherer, 2001
Hard cheese	1.5	England & Wales	Greenwood *et al.,* 1991
Hard cheese	4	Italy, Germany, Áustria & France	Rudolf & Scherer, 2001
Ice cream	0.5	Finland	Miettinen *et al*., 1999a

3) raw unpasteurized milk is often consumed directly by dairy producers and their families, farm employees and their families, neighbours, etc. Interestingly, a study by Headrick *et al*. (1997) showed that people with less than a high school education were more likely to consume raw milk than those who had completed high school, suggesting that level of education may influence a person's choice to consume raw milk.

4) raw unpasteurized milk is consumed directly by a much larger segment of the population via consumption of several types of cheeses including traditional cheeses manufactured from unpasteurized raw milk. According to Van Kessel *et al*. (2004) the group of people that consume non pasteurized milk or milk products is growing. They are sometimes consumed for practical reasons (e.g. dairy farm families), cultural reasons (e.g. soft Mexican-style cheeses), or for perceived health benefits of natural and unprocessed food.

5) entry of foodborne pathogens via contaminated raw milk into dairy food processing plants can lead to persistence of these pathogens in biofilms and subsequent contamination of processed food products. According to Salo *et al*. (2006) all microbes have a tendency to form microbial cell clusters, so-called biofilms, under suitable conditions; some microbes just have a higher natural tendency to produce a biofilm than others. According to the literature (Salo *et al*., 2006), biofilm problems in the dairy plants have been found in air-handling systems, cooling systems, milk transfer lines on conveyors, in packaging machines, in heat exchangers, on ultra-filtration surfaces, in mixers, tanks and other equipment, on floors, and in drains. Common *Listeria* sources in processing plants are conveyor belts, cutters, slicers, brining and packaging machines, coolers and freezers as well as floors and drains (Salo *et al.*, 2006). *L. monocytogenes* growing in biofilms formed in the dairy environment can even contaminate the end product. It has also been found that biofilm cells of *Listeria* were more resistant than planktonic cells to disinfectants containing, e.g. chlorine, iodine, quaternary ammonium and anionic acid compounds (Salo *et al*., 2006). It has also been shown that *L. monocytogenes* can attach not only to stainless steel surfaces but also to rubber, glass, and polypropylene and grow there as a biofilm (Waak *et al*., 2002). Arizcun *et al*. (1998) investigated decontamination procedures to remove *L. monocytogenes* growing in biofilms on glass surfaces. A time-temperature treatment of 63 ºC for 30 minutes resulted in a decline of 5.5 log units in biofilm population.

6) pasteurization may not destroy ALL foodborne pathogens in milk (Oliver *et al.,* 2005)**, and**

7) faulty pasteurization will not destroy all foodborne pathogens (Oliver *et al.,* 2005)**.**

II.2.1 *L. monocytogenes* relation with the dairy indutry

The ability of *L. monocytogenes* to survive and proliferate well in foods stored at refrigeration temperatures makes this organism a particular concern for the dairy food industry, since low initial contamination levels (possibly even at < 1cfu/25g) may increase to numbers that could present a human health hazard if products are subject to

extended refrigerated storage (Wiedmann, 2003). Consequently, humans appear to be commonly exposed to *L. monocytogenes* by food ingestion and the long-term effects of continued exposure to milkborne pathogens on human health are virtually unknown (Jayarao & Henning, 2001).

Based on the FDA/USDA Draft (2001) *L. monocytogenes* risk assessment (Food and Drug Administration and US Department of Agriculture, 2001), the average US consumer is likely to occasionally (possibly once a year) consume as many as 10^6-10^9 cfu of *L. monocytogenes* in a single serving. In spite of this apparent occasional high exposure, only 2,500 human listeriosis cases occur annually in the United States (Wiedmann, 2003).

II. 2.1.1 Situation in Europe

The consumption of dairy products in EU countries is also substantial, averaging 132 Kg per person annually. Milk and other dairy products, are consumed by all age groups, including those populations at risk for contracting listeriosis (European Comission, 2000). Dairy products have been associated with approximately half of the reported listeriosis outbreaks in Europe. Investigated outbreaks (associated with dairy products) to date have resulted in almost 400 cases and over 60 fatalities in Europe (table 2) (Lundén *et al*., 2004).

Table 2: Reported listeriosis outbreaks in Europe caused by milk or dairy products (*in* Lundén *et al*., 2004).

Year	Country	Product type	# of cases (deaths)	Serotype	Reference
1949-1957	Germany	Raw milk	≈ 100	NA [1]	Seeliger, 1961
1983-1987	Switzerland	Soft cheese[2]	122 (33)	4b	Büla *et al*., 1995
1986	Áustria	Raw milk/vegetables	28 (5)	1/2 a	Allenberger & Guggenbichler, 1989
1989-1990	Denmark	Blue-mold cheese/hard cheese	26 (6)	4b	Jensen *et al*., 1994
1995	France	Soft cheese [2]	37 (11)	4b	Goulet *et al*., 1995; Rocourt *et al*., 1997
1997	France	Soft cheese [2]	14	4b	Jacquet *et al*., 1998
1998-1999	Finland	Butter [2]	25 (6)	3a	Lyytikäinen *et al*., 2000
2001	Sweden	Soft cheese [2,3]	33	1/2 a	Carrique-Mas *et al*., 2003

[1] Data not available
[2] Vehicle of infection identified.
[3] Mixed etiology possible.

About 0.2-0.8 cases of listeriosis per 100,000 persons occur annually in developed countries. This results in 1,600-8,400 cases in Europe per year with 320 – 2,500 deaths (table 3).

Table 3: Incidence of listeriosis cases in some European countries (adapted from Lundén *et al*., 2004).

Country	Incidence per 100,000 persons	Year	Reference
England & Wales	0.2	1994-1996	Anonymous, 1997
Iceland	0.2	1996-2000	Anonymous, 2001
Finland	0.4	2000-2002	National Public Health Institute, 2003
Norway	0.4	1996-2000	Anonymous, 2001
Sweden	0.5	1996-2000	Anonymous, 2001
France	0.5	1997	Goulet *et al*., 2001
Latvia	0.7	2002	Public Health Agency, 2002
Denmark	0.8	1996-2000	Anonymous, 2001
Austria	0.24	2004	Rossmanith *et al*., 2006

The wide range in the incidences may result because of differences in the notification systems or due to outbreaks, which may markedly increase the number of cases. The number of reported cases related to outbreaks during 1991-2001 in Europe was 2065. It should be emphasized that some of the sporadic cases may have in fact been part of unrecognized outbreaks. The noninvasive disease form and possible noninvasive disease outbreaks are also probably underdiagnosed because *L. monocytogenes* is not routinely screened from stool samples and the syndrome is usually self-resolving (Lundén *et al.*, 2004).

II.2.1.2 Situation in Portugal

In Portugal, Listeriosis is not a notifiable infection (in contrast with what happens, for example, in Finland and France, where notification is mandatory (Lundén *et al*., 2004)) and available data are scarce (Almeida *et al*., 2006). The real situation regarding listeriosis in Portugal is unknown, and little data exist on both the incidence of this infection or the prevalence of *L. monocytogenes* in foods consumed in the country (Vaz-Velho *et al*., 2000; Guerra *et al*., 2001). In a study by Guerra *et al*. (2001), 54 samples of cows raw milk were analised, with 3 positive for *L. monocytogenes.* In a study by Mena *et al*. (2004), 6 raw milk samples were analised, with one being positive for the presence of *L. monocytogenes.* The study by Almeida *et al*. (2006), concluded that for the period between 1994 and 2003 inclusive, 35 cases of listeriosis were identified, with a mortality rate being greater than 17% (Table 4).

Table 4: Listeriosis in Portugal: 1994-2003 (*in* Almeida *et al*., 2006).

Date of isolation	Age/Sex	Predisposing Factors	Clinical manifestations	Isolated from	Clinical outcome
November, 1994	New-born/M	Age	DNR	Liver	Fatal
1996	DNR/M	AIDS	Fever	Blood and CSF	DNR
July, 1997	DNR	DNR	DNR	Blood	DNR
September, 1997	DNR	DNR	DNR	CSF	DNR
October, 1997	DNR	DNR	DNR	CSF	DNR
1998	54/M	DNR	DNR	Blood	DNR
April, 1998	DNR	DNR	DNR	CSF	DNR
January, 1999	DNR	DNR	DNR	CSF	DNR
July, 1999	DNR	DNR	DNR	CSF	DNR
September, 1999	DNR	DNR	DNR	Blood	DNR
October, 1999	New-born/F	Age	DNR	Lung	Fatal
2000	48/M	DNR	Fever and headache; Stiff neck	CSF	DNR
2000	25/F	DNR	Flu síndrome 15 days before birth	Vaginal culture	Birth at 36 weeks of pregnancy
2000	New-born/DNR	Age	Hypotonia; breath difficulties; sepsis at birth; Apgar score 5 (1 min) – 7 (5min)	Blood	Favourable without sequelae
March, 2000	DNR	DNR	DNR	Blood	DNR
April, 2000	DNR	DNR	DNR	Blood	DNR
June, 2000	DNR	DNR	DNR	CSF	DNR
October, 2000	DNR	DNR	DNR	Blood	DNR
May, 2001	41/M	Cirrosis	DNR	Blood	Fatal
October, 2001	85/M	Age	DNR	Blood	Fatal
February, 2002	DNR/M	DNR	Meningitis	CSF	DNR
March, 2002	55/M	Haematological illness	DNR	Blood	Favourable
2003	75/F	Chronic renal failure	DNR	Blood	Favourable
2003	New-born/ DNR	Age	DNR	Blood	Favourable
2003	DNR/F	DNR	DNR	Vaginal culture	DNR
2003	DNR/F	DNR	DNR	Vaginal culture	DNR
January, 2003	69/M	Age	DNR	Blood	Favourable
February, 2003	74/F	Age	Meningitis	CSF	Favourable
February, 2003	31/F	Pregnancy	DNR	Placenta	Favourable
April, 2003	New-born/DNR	Age	DNR	Blood	Favourable without sequelae
April, 2003	67/M	Corticosteroid therapy; Nephritic síndrome	Meningitis	Blood, CSF and ascitic fluid	Fatal (septic shock)
May, 2003	25/M	DNR	Meningitis	CSF	DNR
July, 2003	85/F	Age	DNR	CSF	DNR
October, 2003	50/M	Alcoholism	DNR	Blood/ CSF	Fatal
November, 2003	48/M	DNR	DNR	CSF	With internment return but without sequelae

DNR: Data Not Recorded
F, Female; M, Male
CSF, Cerebrospinal fluid

II.2.2 Summary

In summary, potential threats to human health related to dairy products and dairy farming include errors in pasteurization, consumption of raw milk products, contamination of milk products by emerging heat-resistant pathogens, emergence of antimicrobial resistance in zoonotic pathogens, chemical adulteration of milk, transmission of zoonotic pathogens to humans through animal contact (e.g. farm workers and visitors), and foodborne disease related to culled dairy cows (Ruegg, 2003).

II.3 HACCP

Consumer concern about livestock production methodologies has been increasing over the last decades due to various outbreaks of food-borne zoonoses and animal diseases. The general public nowadays has little knowledge about agro-production. Its perception is largely determined by the calamities that occur and that attract media attention. At the same time, the dairy industry is highly susceptible to incidents affecting the public image of their products (Noordhuizen & Metz, 2005).

Quality assurance programmes in the different production chains have been installed by industry to counteract the problems occurring. The primary producers, like the dairy farms, are not formally included in such programmes. Yet, quality control at dairy farm level goes beyond the quality control of the product milk alone. “Quality” can no longer be associated with the product alone, but should be extended to the production process itself. For better safeguarding food safety and public health, as well as animal health and welfare the whole production process on the dairy farm should be addressed. Today, consumers have quite an impact on animal production in Europe especially regarding the husbandry system, animal health care and animal transportation. The European Comission has prioritized consumer protection in its policy, installed the precautionary principle, and created the European Food Safety Authority, EFSA. A directive (97-12) and regulation (178-2002) point to the need to monitor farms for food safety, public health, animal health and welfare.

Based on developments within the dairy sector as well as at the European Union (EU) political level, it can be expected that the application of HACCP-compatible programmes on the dairy farms will be conducted in the near future. This application will help to identify and manage the quality hazards and risks occurring in the

production process on dairy farms, and in providing the consumer with more certainty about the quality of products of animal origin.

Several food production sectors have already implemented integrated quality assurance programmes throughout the whole chain, including the farms. Examples are the cattle quality management programmes in Australia, Scandinavia, and Canada.

II.3.1 HACCP: Is it coming to the dairy?

The HACCP concept is, according to Noordhuizen & Metz (2005), the best choice if a quality control programme should be designed for dairy farms. Particularly because it is highly farm-specific, easy to link up with operational management, relatively low in cost, both product and process oriented, and not requiring much labor. In any case, a sound quality attitude of farmers and others involved is needed before one should even think about introducing HACCP. The HACCP concept deals with hazard and risk identification, process decomposition, designation of critical control points, the set-up of an on-farm monitoring programme, the documentation and the verification of the programme.

Food safety, public health, animal health and animal welfare should, according to the same authors, be integrated into one HACCP-based programme because (1) disorders (the hazards) in any of the four areas are predominantly multicausal in nature, (2) hence focus must be on risk identication and risk management, (3) HACCP principles comprise such hazard and risk identification, (4) the process of production can be brought under control more efficiently and (5) therefore the product quality can be assured more effectively than by separate approaches of each aspect. It is quite possible that HACCP-based programmes, building on good manufacturing codes, will become compulsory for dairy farmers, as as been said, in given EU member states or regions within a few years.

These ideas are not, however, the opinion of all the authors. For example, Ruegg (2003) thinks that there are several aspects of HACCP that make widespread adoption on dairy farms unlikely. Specifically, because HACCP programs require critical multidisciplinary review of existing management processes, the establishment of limits via identification of critical control points, the use of routine surveillance procedures, effective record keeping, and documentation of standard processes. The technology to carry out on-farm HACCP programs is further limited by inadequacies and costs of

existing testing methodologies (Gardner, 1997). These limitations have led some to abandon farm-level HACCP programs in favor of alternative approaches such as "Hurdle Technology" (Heggum, 2001).

II.3.2 Possible alternative - Hurdle technology

Hurdle technology refers to the application of a combination of selected "hurdles" to microbial growth combined with processing steps that maintain and improve the microbial stability and sensory quality of foods (Heggum, 2001). Hurdles commonly used in food processing are directed at reducing growth of microrganisms present on harvested food products and include chilling, alteration in pH, the use of competitive microorganisms and alterations in water content (Leistner, 2000). The basic concept of hurdle technology is to produce an environment that is hostile to the growth of microorganisms (Ruegg, 2003).

The production of nonfermented dairy products does not include mitigating factors or hurdles to the growth of *Listeria* as effective as those seen for fermented products. There are, however, some processes which have been shown to reduce the population of *Listeria*. One such process is separation for the adjustment of milkfat content. These centrifugal processes tend to decrease the levels of *Listeria* in skimmilk, lowfat milk, half and half, cream, and butter. These processes are particularly effective in removing *Listeria* if leukocytes containing the organism are still present after initial clarification of the milk. This effectiveness is a result of the separator continuing to act as a clarifier by removing somatic cells (Kozak *et al*., 1996).

The concept of on-farm hurdles may be extended to include best management practices (BMP) focused on the exclusion of bacteria from raw milk supplies. The next section refers some examples of BMP and their relation with food safety.

II.3.2.1 Best management practices

Many farmers are currently using hygienic milking practices and the effective use of predipping and forestripping has been shown to enhance milk safety. The use of predipping using iodine has been demonstrated to reduce standard plate counts and coliform counts in raw milk by five-and six-fold, respectively, as compared to other methods of premilking udder preparation (Galton *et al*., 1986). The overall reduction of

microbial loads in raw milk through the use of predipping should result in reduced numbers of zoonotic pathogens. Predipping has been shown to reduce the risk of *L. monocytogenes* in milk filters by almost four-fold (Hassan *et al*., 2001). The examination of milk before attaching milking units is necessary to ensure that all abnormal milk is diverted from the human food chain and should be a standard food safety practice on ALL farms. Similar to predipping, the use of forestripping has been shown to significantly reduce (2.5 times less likely) the risk of contamination of milk with *L. monocytogenes* (Hassan *et al*., 2001).

Excellent hygienic standards for housing and milking centers and cleanliness of cows result in reduced opportunities for growth and transmission of pathogenic bacteria. A study by Sanaa *et al*. (1993) found that, besides silage of poor quality (pH>4), inadequate frequency of cleaning the exercise area, poor cow cleanliness, insufficient lighting of milking barns and parlors, and incorrect disinfection of towels between milkings were significantly associated with milk contamination by *L. monocytogenes*. Commonly used food plant sanitizers (chlorine, acid anionics, quaternary ammonium compounds, and iodophors) are effective against *Listeria* but only when applied to clean surfaces, i.e., all organic material must be removed from surfaces before using sanitizers (Pearson & Marth, 1990).

II.3.3 Summary

Most dairy farmers feel responsible for the safety of milk and beef that originate on their farms, but linkage between farm production practices and the quality of processed products have been weak.

The universal implementation of these interventions is a major challenge for the complex and highly diverse dairy industry. Virtually all dairy producers must understand the linkage between animal management and the safety and quality of food products. It is unlikely that education alone will motivate dairy farmers to voluntarily adopt practices for which they don't recognize an immediate economic return, and so, in my opinion, the application of HACCP plans to the Portuguese dairy farms will be a difficult task.

III. GENUS LISTERIA

In this chapter, the genus Listeria will be reviewed. First, a historical overview and then the main genus characteristics, including culture, temperature, pH and water activity (a_w) limits, metabolism and biochemical characteristics, as well as classification, virulence and serotype distribuition in Europe.

III. 1 Historical Overview

Unlike some pathogenic agents responsible for large outbreaks which have marked the history of humans for centuries, for example, *Vibrio cholerae* or *Yersinia pestis*, the history of *L. monocytogenes* and listeriosis is recent: It began officially in 1924. The first confirmed diagnosis in a human was that of a soldier suffering from meningitis at the end of World War I, and before this case, there are no validated observations. Interestingly, however, a historian has suggested that *L. monocytogenes* could have been the cause of Queen Ann's 17 unsuccessful pregnancies (17^{th} century) (Rocourt, 1999).

When in 1924 E.G.D. Murray isolated Gram-positive rods from the blood of laboratory animals (rabbits), he could not assign these pathogenic microorganisms to any bacterial genus known at that time. Thus, he called this new agent *Bacterium monocytogenes.* It can be anticipated that, even before Murray, other bacteriologists had already grown this particular bacterium without having a clear classification (Hof, 2003).

In 1928, Matthews detailed an outbreak of encephalitis of unknown origin in cattle which, in retrospect, was probably bovine listeriosis. Nyfeldt in 1929 reported the incidence of the first human case of listeriosis (Dhanashree *et al.*, 2003). Listerial encephalitis has since been well documented.

In 1940 Pirie named the genus *Listeria* for catalase-positive, Gram-positive rods. This had become necessary, because in the meantime such bacteria had been isolated in some other cases from humans, from animals as well as from food and the environment. These incidental observations did not, however, establish a broad awareness about this pathogen in the community of infectious disease specialists, microbiologists, or food microbiologists.

The particular role of this type of bacteria as a pathogen was not realized until an epidemic of listeriosis in newborns occurred in Germany in 1949. In the Institute of Pathology of the University of Halle, a peculiar entity was observed, hitherto unknown and called "granulomatosis infantiseptica". In 85 newborns or stillborn infants, granulomas were detected histopathologically in various organs such as liver, spleen, brain, lung and skin. A young bacteriologist, J. Potel, was able to isolate bacteria from meconium, blood or various organs; he classified them in the genus *Corynebacterium*. At about the same time similar cases of connatal infections were observed and studied at the University of Bonn. The bacteria isolated from these lesions were examined by H.P.R. Seeliger. He detected the motility of these pathogens, which was not consistent with *Corynebacteria* but rather with *Listeria* (Hof, 2003).

At that time a new era of research on listeriosis started. In the following years Seeliger invested an enormous effort to inform the public about *Listeria* and listeriosis. He compiled the first overview in the book "Listeriosis", nearly a biblical publication.

The role of *Listeria* in mastitic infections was not clearly identified until 1944 when Wramby isolated *L. monocytogenes* from milk and udders of mastitic cows in Sweden. Before this, in 1938, Schmidt and Nyfeldt had postulated that a small outbreak of human listeriosis in Denmark may have been caused by drinking milk from mastitic cows. In 1956, de Vries and Strikwerda described another case of bovine mastitis in which a penicillin-resistant strain of *L. monocytogenes* was cultured from one quarter of a 6-year-old dairy cow (Wesley, 1999). Following acute onset, the condition soon became chronic with shedding of *L. monocytogenes* in milk for 3 months.

Although, as already was said, the incidence of the first human case of listeriosis was reported by Nyfeldt in 1929, it was only since 1981, after the three well-investigated listeriosis epidemics (one caused by coleslaw (Schlech *et al*., 1983), second caused by whole and 2% milk (Fleming *et al*., 1985) and a third caused by consumption of soft Mexican-style cheese (Linnan *et al*., 1988), that this organism came to be considered as a foodborne pathogen (Dhanashree, 2003).

Today, there are at least four major fields of interest in *Listeria (*Hof, 2003*)*:

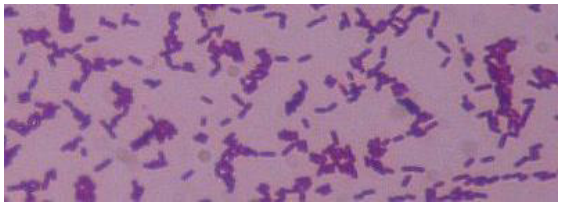
1) the role in medical microbiology: *L. monocytogenes* causes severe diseases of humans and animals and is difficult to treat and diagnose;
2) the role in food microbiology: *Listeria* is a food-borne pathogen and is found in various food items;

3) the role in cell biology: *L. monocytogenes* is a facultative intracellular parasite having an intense cross-talk and interactions with the host cell; and,

4) the role in immunology: basic knowledge on cell-mediated immunity has been acquired using listeriosis as a model.

III. 2 Genus characteristics

Listeria is a small (0.5 µm in diameter and 1-2 µm in length), regular Gram-positive rod with rounded ends (fig. 1). Cells are found singly, or in short chains, or may be arranged in V and Y forms or in palisades. Sometimes cells are cocoid, averaging about 0.5 µm in diameter and may be confused with streptococci. In old cultures, some cells lose the ability to retain the Gram stain and may be occasionally mistaken for *Haemophilus. Listeria* does not produce spores and capsules are not formed (Rocourt, 1999).



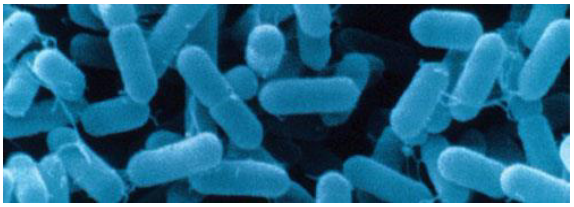


Fig. 1: *Listeria* Gram stain (left) *in* http://cdl.niedersachsen.de and scanning EM (right) *in* http://textbookofbacteriology.net

Listeria is motile because of its few peritrichous flagella (fig. 2) when cultured at 20-25 ºC (not or very weakly motile at 37 ºC). Hanging-drop preparations of fresh cultures in tryptose phosphate broth incubated at 20 ºC show characteristic tumbling motility: cells start with twisting and wriggling movements which increase to fast, eccentric rotations before they suddenly move quickly in various directions. Stab cultures in semisolid motility medium produce a typical picture of "umbrella" or inverted "pine tree" growth about one half centimeter below the surface. (Rocourt, 1999).

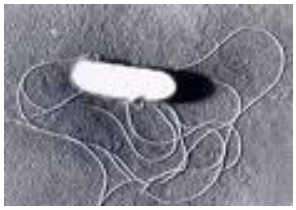


Fig. 2: *L. monocytogenes* scanning EM, showing flagella *in* *http://textbookofbacteriology.net*

III. 2.1 Culture

On nutrient agar, colonies are 0.2-0.8 mm in diameter, smooth, punctiform, bluish gray, translucent, and slightly raised with a fine surface texture and entire margin after 24 h of incubation. After 5-10 days, well-separated colonies may be 5 mm or more in diameter. When cultures of *Listeria* grown for 18-24h at 37 ºC on a clear medium are examined with a binocular microscope under obliquely transmitted light, the smooth colonies exhibit a typical blue-green iridescence.

Listeria usually grows well on most commonly used bacteriological media (Rocourt, 1999). Polymixin Acriflavine LiCl Ceftazidime Aesculin Mannitol Agar (PALCAM) and Agar Listeria Ottavani & Agosti (ALOA) were the two bacteriological media used in this study.

PALCAM agar provides a quantitative cultivation of *L. monocytogenes*, while, at the same time, inhibiting the Gram-negative and most of the Gram-positive accompanying bacteria. The selectivity of the medium results from its content of polymyxin, acriflavin, ceftazidime and lithium chloride. *L. monocytogenes* breaks down the esculin in the medium to glucose and esculetin. Esculetin forms an olive-green to black complex with iron (III) ions which stains the colonies of *L. monocytogenes* (fig. 3). Mannitol-positive accompanying bacteria such as staphylococci grow as yellow colonies, if they are not inhibited.

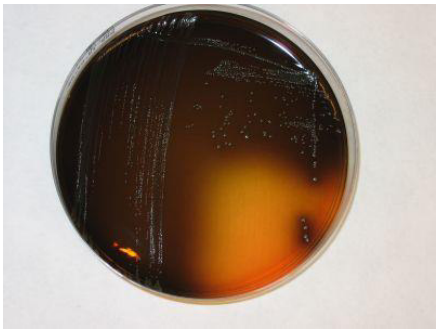


Fig 3. *L. monocytogenes* on Palcam selective agar medium.

On the other hand, ALOA agar is a pre prepared, selective and differential medium for the isolation of *Listeria* spp. from food samples and for the presumptive identification of *L. monocytogenes*. To minimise the growth of contaminating organisms, lithium chloride and a balanced antimicrobial and antifungal mixture is employed. The incorporation of the chromogenic substrate X-glucoside for the detection of beta-glucosidase demonstrates the presence of *Listeria* spp., whilst the detection of a specific phospholipase C enzyme produced by pathogenic *Listeria* spp. including *L.*

monocytogenes is also achieved. *Listeria spp*. grow on this medium producing blue - green colonies, with pathogenic species (*L. monocytogenes* and *L. ivanovii*) producing similar coloured colonies surrounded by a characteristic opaque halo after 24 hours incubation at 37 ºC (fig. 4). Non *Listeria* spp. produce white colonies.

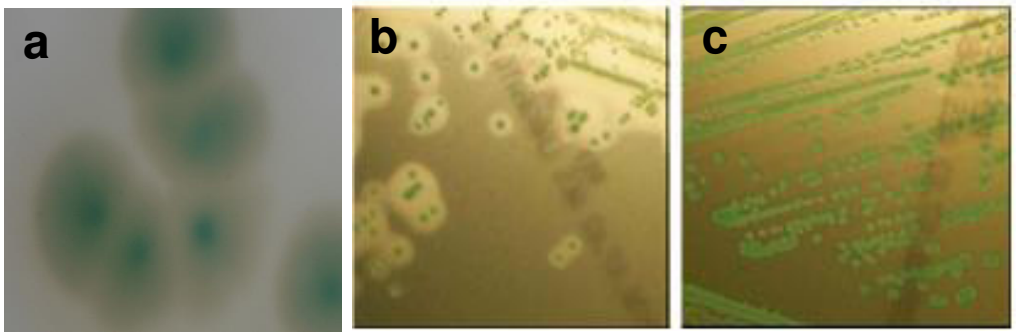


Fig. 4: *Listeria* spp. typical colonies on ALOA medium.

Legend:

a - Typical colonies of *L. monocytogenes* (photos of colonies isolated in this study)

b - Typical colonies of *L. ivanovii* (gently offered by the ESB-UCP lab.)

c - Typical colonies of *L. innocua* (gently offered by the ESB-UCP lab.)

Although the development of the opaque halo around colonies of *L. monocytogenes* is highly characteristic, *L. ivanovii* also produce the specific phospholipase C responsible for this halo. Therefore, in order to accurately report the presence of *L. monocytogenes*, specific identification must be performed, as will be described in section VII.3.

III. 2.2 Temperature, pH and a_w limits

The normal temperature limits for growth are +1-2 ºC to 45 ºC (Rocourt, 1999), and is thus considered psychotrophic. All *Listeria* spp. are heat sensitive and pasteurization effectively kills listeria (Kozak *et al*., 1996; Hassan *et al*., 2001; Muraoka *et al*., 2003; Wiedmann, 2003; Nightingale *et al*., 2004;).

Listeria normally grows from pH 4.4-9.6, optimally at pH 7. Growth can occur in media containing 10% (w/v) NaCl with survival occuring at higher concentrations. Survival at low pH and high salt concentration is strongly temperature-dependent. It should be emphasized that *Listeria* is one of the few foodborne pathogens that can grow at an a_w below 0.93 (Farber *et al.,* 1992).

Perhaps due to its considerable resistance to a variety of stress conditions, *L. monocytogenes* appears to be able to multiply and/or survive for extended periods (up to more than 2 years) outside mammalian hosts. Consequently it has been isolated from a variety of different environments (e.g. soil, surface water, sewage, vegetative materials, different food processing environments), and many authors thus consider this organism "ubiquitous" (Wiedmann, 2003).

III. 2.3 Metabolism and Biochemical Characters

All *Listeria* species are catalase positive, oxidase negative, and aesculin hydrolysis positive. The members of the genus are aerobic, but also grow under anaerobic conditions and can thus be classified as facultative anaerobes (Wiedmann, 2003); growth is enhanced in an atmosphere with reduced oxygen and 5-10% CO_2 (Pearson & Marth, 1990).

III. 3 Classification

The genus *Listeria* belongs to the *Clostridium* subbranch, together with *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Brochothrix*. This phylogenetic position of *Listeria* is consistent with its low G+C DNA content (36-42%) (Allerberger, 2003). In addition to *L. monocytogenes* the genus *Listeria* also includes 5 other species: *Listeria ivanovii, Listeria seeligeri, Listeria innocua, Listeria grayi and Listeria welshimeri* (fig. 5)

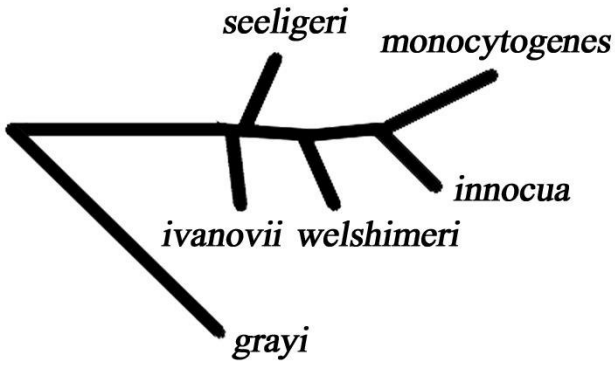


Fig. 5: Taxonomic relatedness of the *Listeria* species based on 16S ribossomal RNA sequence data (*in* Bell & Kyriakides, 1998).

Whereas *L. monocytogenes* causes both human and animal disease, *L. ivanovii* is predominantly associated with disease (specifically abortions) in sheep, and human clinical infections with this organism appear to be extremely rare (Wesley, 1999). The other *Listeria* species are considered non-pathogenic. The pathogenic species *L. monocytogenes* and *L. ivanovii* are both haemolytic, as is the non-pathogenic *L. seeligeri,* while *L. innocua* and *L. welshimeri* are nonhaemolytic.

L. monocytogenes is grouped into 13 serotypes based on the O and H antigens (Yoshida *et al*., 1999): 1/2a, 1/2b, 1/2c, 3a,3b,3c,4a, 4ab, 4b, 4c, 4d, 4e and 7, all of which appear to have been associated with animal disease. Generally serotypes 1/2a, 1/2b, and 4b appear to be most common among animal isolates, although serotype 4c is also occasionally found. *L. monocytogenes* serotypes 1, 1/2a, and 4b have been reported to occur in raw milk (Jayarao & Henning, 2001).

There are some indications that the frequency of different serotypes and molecular subtypes among human and animal cases differs. Thus, certain subtypes may show at least some level of host specificity for humans and animals (Wiedmann, 2003). Data from a study made by Nightingale *et al*. (2004) suggest that some *L. monocytogenes* subtypes may be adapted to infect mammalian hosts, some may be adapted to environmental survival, and others may be equally adapted to ruminant hosts and environmental survival and thus, may be particularly successful at surviving by establishing high population densities in multiple niches.

III.3.1 According with virulence

L. ivanovii and *L. seeligeri* contain **virulence** genes that are highly similar to those found in *L. monocytogenes*. Despite the presence of *L. monocytogenes* virulence gene homologues in *L. seeligeri*, this species is considered non-pathogenic and has been shown to be avirulent in the murine animal model. *L. innocua* and *L. welshimeri* completely lack the virulence gene homologues found in *L. ivanovii*, *L. seeligeri* and *L. monocytogenes* (Wiedmann, 2003).

Virulence of *L. monocytogenes* may be related to two toxins: hemolysin and cytotonic. The hemolysin is a cytolysin able to lyse tissue and red blood cells. The cytotonic toxin stimulates cyclic AMP production similar to cholera toxin. All serotypes

of *L. monocytogenes* have the ability to provoke **monocytosis** (Pearson & Marth, 1990), fact that originated its name (table 5).

Table 5: Members of the genus *Listeria* (*in* Bell & Kyriakides, 1998), and their names origin.

Listeria species	Previous species names	Origin of species name
Listeria monocytogenes	*Bacterium monocytogenes*	Increased monocyte production
Listeria innocua		Innocuous/harmless
Listeria welshimeri		After H.J. Welshimer, American bacteriologist
Listeria seeligeri		After H.P.R. Seeliger, German bacteriologist
Listeria grayi	*L. grayi, L. murrayi* (in honor of E.G.D. Murray, a Canadian microbiologist)	After M.L. Gray, American bacteriologist
Listeria ivanovii **Subspecies *ivanovii*** **Subspecies *londoniensis***	*L. ivanovii* *L.monocytogens* serovar 5	After I. Ivanov, Bulgarian bacteriologist

Virulence gene allelic analysis, ribotyping and comparative virulence characterization have been shown to subdivide *L. monocytogenes* into three lineages. Lineage I consists of strains (flagellar antigen types b and d) that are more likely to cause human than animal disease than isolates classified into linages II (antigen type a or c) and III (rarely detected serotypes, 4a and 4c) (Cabrita *et al*., 2004), and also appear to show an increased ability to spread intracellularly from host cell to host cell (Wiedmann, 2003).

Based on the variable gene content, the three lineages I, II and III of *L. monocytogenes* were further divided into five phylogenetic groups, each correlated with serovars: I.1 (1/2a-3a), I.2 (1/2c-3c), II.1 (4b-4d-4e), II.2 (1/2b-3b-7), and III (4a-4c) (Doumith *et al*., 2004).

III.3.2 Serotype distribution in Europe

The most common serotype in **European listeriosis outbreaks** has been **4b** (Lundén *et al.,* 2004). The distribution of serotypes in outbreaks caused by vehicles other than dairy products appears to be similar to that of dairy product-linked outbreaks

(table 6). However, there is a discrepancy between clinical isolates and food isolates as serotype 4b is not the most common serotype in food isolates (Lundén *et al.,* 2004). The serogroup **1/2** is the leading serogroup in **foods** (Lundén *et al.,* 2004).

Table 6: Reported *Listeria* outbreaks in Europe according to food type (*in* Lundén *et al*., 2004).

Food type	Year	Country	Serotype	Reference
Dairy	1949-1957	Germany	NA[1]	Seelinger, 1961
	1983-1987	Switzerland	4b	Büla *et al*., 1995
	1986	Áustria	1/2a	Allenberger & Guggenbichler, 1989
	1989-1990	Denmark	4b	Jensen *et al*., 1994
	1995	France	4b	Goulet *et al*., 1995
	1997	France	4b	Jacquet *et al*., 1998
	1998-1999	Finland	3a	Lyytikäinen *et al*., 2000
	2001	Sweden	1/2a	Carrique-Mas *et al.,* 2003
Meat	1987-1989	United Kingdom	4b	McLauchlin *et al.,* 1991
	1992	France	4b	Goulet *et al*., 1993; Jacquet *et al*., 1995
	1993	France	4b	Goulet *et al.,* 1998
	1999-2000	France	4b	De Valk *et al*., 2001
Fish	1994-1995	Sweden	4b	Ericsson *et al*., 1997
	1997	Finland	1/2a	Miettinen *et al.,* 1999b
Vegetables	1993	Italy	1/2b	Salamina *et al*., 1996
	1997	Italy	4b	Aureli *et al.,* 2000

[1]Data not available

III.4 *L. monocytogenes* regulations

Current regulations specifying a zero-tolerance (in the US), and absence in 25 g (EU regulation 2073/2005), for the presence of any *L. monocytogenes* subtypes in ready-to-eat foods are based on historical taxonomic classification schemes. These classical taxonomic definitions of bacterial species do not necessarily correlate with the ability of a group of bacteria to cause human disease. Rather, as outlined above, related bacteria that differ in their abilities to cause human and/or animal disease may be grouped together into the same species. Thus a critical need exists for the development of better scientific definitions of bacterial groups that have the ability to cause human disease. Molecular subtyping methods provide a unique opportunity to explore the population genetics and evolution of *L. monocytogenes*. Understanding bacterial diversity beyond the species level thus represents an important task for all food and dairy microbiologists (Wiedmann, 2003).

IV. MASTITIS

In this chapter the basic topics about mastitis will be refered, including the different aetological agents. Particular emphasis will be given to the relationship between mastitis and food safety, the "no growth" culture results and listerial mastitis.

Mastitis, milk quality and dairy food safety are all very much interrelated. Mastitis, an inflammation of the mammary gland caused by (usually) bacterial infection, trauma, or injury to the udder, remains the most common and most expensive disease affecting dairy cattle throughout the world. Mastitis is caused by several different bacteria that can invade the udder, multiply there and produce harmful substances that result in inflammation, and continues to be one of, if not, the most significant limiting factor to profitable dairy production (Oliver *et al*., 2004).

Mastitis reduces milk yield and alters milk composition. The most notable **changes in milk composition associated with mastitis are**:

- decreased concentration of: fat, lactose, casein and calcium, and
- increased concentrations of: albumin, sodium and chloride. Concentrations of enzymes such as lipases, proteases, oxidases, plasmin and plasminogen also increase and this may adversely influence milk stability, milk flavour and processed dairy products.

Mastitis can also be divided into **clinical** and **sub-clinical**, according with milk aspect. Clinical mastitis is characterized by abnormal milk and/or visible abnormalities of the udder such as hot and swollen udders. However, subclinical infections, the most common form of mastitis, are not readily apparent because there are no visible signs of the disease.

The measurement used most commonly to detect subclinical mastitis is the somatic cell count (SCC) of milk. One characteristic feature of mammary gland inflammation is an elevation in the number of somatic cells in milk. Milk from uninfected mammary glands contains < 100,000 somatic cells per millilitre. There is ample evidence that increased prevalence of subclinical mastitis in a dairy herd (as demonstrated by high Bulk Tank Somatic Cell Count (BTSCC)) is indicative of management practices associated with reduced food safety. Monthly BTSCC values were higher in herds where verotoxigenic *E. coli* and *L. monocytogenes* were cultured from bulk tanks as compared to herds negative for those pathogens in a study by Steele *et al.* (1997). In contrast, Hassan *et al*. (2000) and Van Kessel *et al*. (2004) did not find

an apparent relationship between SCC and *Salmonella* or *L. monocytogenes* contamination.

IV. 1 Aetiological agents

Lactococcus, Lactobacillus, Streptococcus, Staphylococcus and *Micrococcus* spp. are the common bacterial flora of fresh milk. Fresh milk drawn from a healthy cow normally contains, as as already been said, a low microbial load (less than 1000/mL). Growth of most microorganisms is favored by the presence of organic compounds, moderate or warm temperatures, adequate oxygen, and neutral pH. All of these conditions are present in the modern dairy farm and are most commonly recognized when mastitis problems develop.

Microorganisms that most frequently cause mastitis can, classically, be divided into two categories: contagious pathogens and environmental pathogens. Contagious mastitis is caused primarily by *Staphylococcus aureus* (fig. 6) and *Streptococcus agalactiae*. The primary source of these organisms is the udder of infected cows. Contagious mastitis pathogens spread from infected cows to uninfected cows primarily during milking.

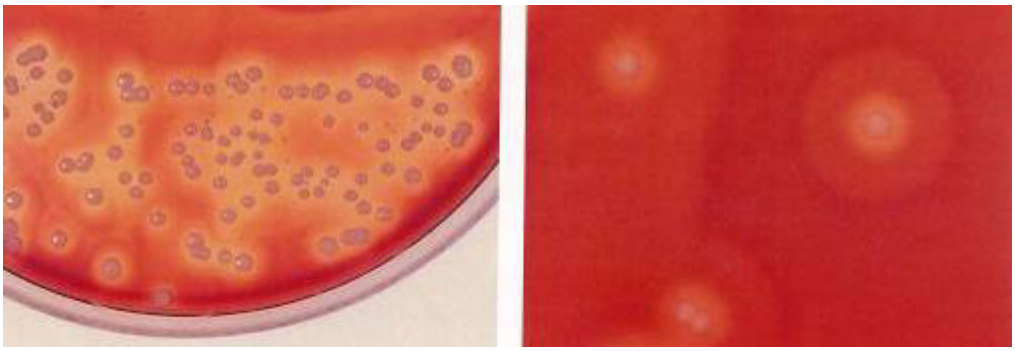


Fig. 6: *S. aureus* colonies in blood agar, showing typical α and β hemolysis.

Environmental mastitis is caused primarily by environmental streptococci and coliforms (fig. 7). The primary source of environmental mastitis pathogens is the environment of the cow.

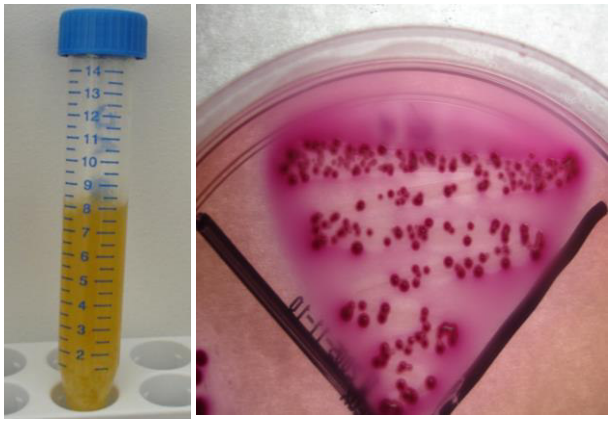


Fig. 7: Typical "beer-like" aspect of a coliform mastitis "milk" sample (left), and *E. coli* on MacConkey agar (right) (photos gently offered by the SVA lab.)

This classification is however being questioned, as the different agents, seem to share characteristics of both categories (Schukken, 2005) (fig. 8), fact that we comprove in our dairy practice.

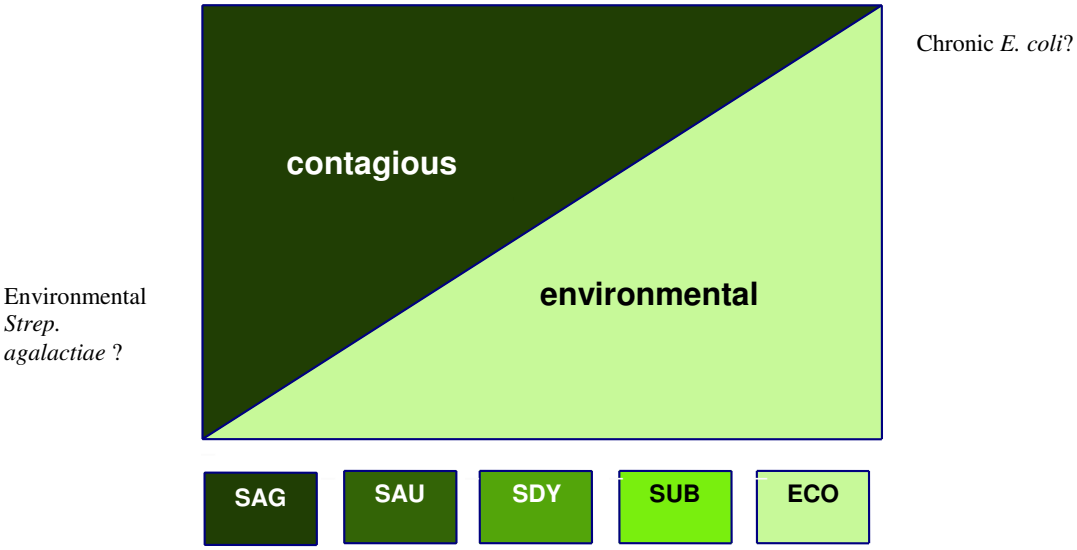


Fig. 8: SAG = *Strep. agalactiae*; SAU = *S. aureus*; SDY = *Strep. dysgalactiae*; SUB = *Strep. uberis*; ECO = *E. coli*. This diagram intends to illustrate that microrganisms of both groups share characteristics, and so, should not be classified in two different isolated groups (adapted from Schukken, 2005).

The importance of various aetiological agents in milkborne disease has changed dramatically over time. Mastitis control programs focusing on hygienic harvesting of milk have been widely adopted for at least 50 years.

Worlwide, farmers have achieved tremendous success in reducing the incidence of contagious mastitis by adopting the five basic principles of mastitis control:

1) postmilking teat disinfection,
2) universal dry cow antibiotic therapy,
3) appropriate treatment of clinical cases,
4) culling of chronically infected cows, and
5) regular milking machine maintenance.

Contagious bacteria, such as *S. aureus* and *Strep. agalactiae*, are now responsible for less than one-third of all mastitis cases compared with more than 75% of all cases 20 years ago (Hillerton *et al.*, 1995).

However, more than 90% of all reported cases of dairy related illness continue to be of bacterial origin, with at least 21 milkborne or potentially milkborne diseases currently being recognized. Pathogens that have been involved in foodborne outbreaks associated with the consumption of milk include *L. monocytogenes, Salmonella, Campylobacter*, *S. aureus, Bacillus cereus* and *Clostridium botulinum* (Chye *et al*., 2004).

IV.1.1 ZOONOTIC AGENTS

The zoonotic agents, briefly described in table 7, are not considered significant mastitis aetiological agents; Either they are not really important, or they are not being looked after as they should be (misdiagnosis). This might be the case of *Listeria* spp., as *Listeria* Gram stain is quite similar with the *Corinebacterium* spp. (a quite common contagious mastitis agent), or even with yeasts. A more detailed description of listerial mastitis will be given in the next section.

Table 7: Causes of mastitis that require public health concerns (adapted from Rebhun, 1995).

Organism	Infection mechanisms of the udder	Clinical signs	Diagnostic	Therapeutic treatment and control measures
Salmonella *Dublin*	Environmental – most probable way: contamination by faeces of carrier asymptomatic cows as a result of septicemic dissemination to the udder.	Usually subclinical or chronic (≥ 6 months). May occur an increase of the Somatic Cell Count (SCC).	ELISA of serum and milk for antibody detection of asymptomatic cows. Culture of milk and faeces.	Detection and culling of asymptomatic animals. Prohibition of raw milk ingestion.
***Salmonella typhimurium and other Salmonella* sp. types (B, C e E)**	Environmental – most probable way: contamination by faeces of carrier asymptomatic cows as a result of septicemic dissemination to the udder.	Usually subclinical. The duration of infection and the factors determining the excretion are not yet established. The excretion may be persistent or intermittent. Animals may present fever and diarrhea, that may present blood. Farm workers may equally become sick.	Culture of milk and faeces.	Prohibition of raw milk ingestion. Control measures may become difficult to establish due to great dispersion of faecal contaminants in the environment.
L. monocytogenes	As a result of septicemic dissemination to the udder. Possible environmental contamination by faeces of animals that have ingested *L. monocytogenes.*	Subclinical signs. Neurological signs. Abortions.	Diagnostic signs only appear in the neurological form. Culture.	Prohibition of raw milk ingestion. Milk pasteurization.
Brucella abortus	As a result of septicemic dissemination to the udder.	Abortions	Serology. Culture.	Prohibition of raw milk ingestion. Intervention of official services.
***Staphylococcus sp* enterotoxin producers**	Contagious or environmental	Subclinical Increase of SCC.	Culture of bulk tank milk.	Long storage periods or inadequate cooling of milk should be avoided. Pasteurization is essential. Prohibition of raw milk ingestion.
Nocardia *asteroides*	Contaminated syringes or intramamamary cannulas. Environmental.	Acute mastitis in fresh cows, with fevers and hardened quarters. Mastitis with subclinical severity in milking cows. Mammary gland fibrosis. Some animals develop piogranulomatous reactions in the affected quarters, and may lead to fistulas and abscesses.	Culture.	Most treatments are rarely efficient. Identification and culling of infected animals. Prohibition of raw milk ingestion.
Cryptococcus neoformans	Contaminated intramammary products.	Acute mastitis with increase of mammary limphatic nodes. Thick white-grey secretions.	Cultures. Smears. Udder biopsy.	Culling of infected cows. Prohibition of raw milk ingestion.

IV.1.1.1 Listerial mastitis

Although not particularly common, generalized **listerial** infections can give rise to **mastitis**. As with sheep and goats, *L. monocytogenes* is also shed in milk by healthy dairy cattle with no indication of mastitis (Gitter *et al.*, 1980) but one infected quarter can shed between 1000 and 10^6 *L. monocytogenes*/mL of milk (Bemrah *et al*. 1998).

In the recent literature the route of infection for naturally occurring cases of listerial mastitis were discussed controversially. Most reports suggested that both a haematogenous and an intramammary route of infection are possible. Bourry *et al*. (1995) considered the intramammary infection to be the most likely and emphasize that the *Listeria* have to contaminate the teat end before penetration into the udder, which is brought on by unhygienic conditions. Data from a study by Winter *et al*. (2004) strongly suggest that naturally occurring *Listeria* mastitis is only caused by penetrating the udder through the teat canal. Interestingly, Chye *et al*. (2004) refer that the presence of bacteria in milk samples may not be due to infection of the udder itself, but arise from the teat duct. According to these authors, the bacteria can be carried into the milk duct of the cow during milking by suction of the milking machine and then flushed out during subsequent milking without causing clinical symptoms of infection.

Prolonged excretion of *L. monocytogenes* in milk, the apparently normal appearance of the milk, and consumption of raw milk on farms could be important factors in the transmission and epidemiology of milkborne listerial infections (Gitter *et al.*, 1980). From a public health aspect, culling of *L. monocytogenes*-infected cows with clinical mastitis which do not respond to treatment is recommended (Sharp, 1989). After slaughter, cross contamination of the carcass with bacteria from the infected udder is possible through evisceration, meat inspection, or other manipulations (Vishinsky *et al.*, 1993).

IV. 2 No growth results

Although all these agents have been recognized as mastitis aetiological agents, reports indicate that 25-40% of all clinical samples are negative on routine culturing.
Reasons to explain this fact include:
-Numbers of certain organisms, such as Mycoplasma, *S. aureus*, and coliforms, can vary greatly in infected quarters, and may occasionally be less than the minimum detection

limit of the assay. The minimum detection limit when plating 0.01 mL of milk is about 100 cfu/mL. Specifically, *L. monocytogenes* has to reach a concentration of 10^5 to 10^7 cfu/mL before the organism can be detected (Hassan *et al*., 2000);

-The organism may no longer be present and the clinical signs are due to by-products such as endotoxins;

-Somatic cells may have phagocytized the organisms;

-Antibiotics may have killed or suppressed organism numbers to unrecoverable levels;

-Storage may have reduced numbers of viable organisms to undetectable levels;

-The organism may require cultural conditions other than those used for isolation (i.e. reduced temperature, prolonged incubation, special media, anaerobic conditions, etc).

One of the main reasons for this "no growth" results are probably *Mycoplasma* spp. The economic impact of mastitis caused by mycoplasma is observed all over the world. In Europe, the estimated cost of the disease can be as elevated as 144 million euros per year (Nicholas *et al*., 2000). In the USA, the cost of the infections caused by the lost in gain and value of the carcasses were estimated in 32 million dollars a year, and 108 million dollars due to mastitis (Rosengarten & Citti, 1999). The disease was first documented in 1961 in the USA (Hale *et al*. 1962) and since then it has been reported in many countries (Rastas & Johnston, 1969; Jasper, 1979; Doherty *et al*., 1994; Aduriz *et al*., 1996; Gonzalez, 1996; Gunning & Shepherd, 1996; Judge, 1997; Byrne *et al*., 2000; Cerdá *et al*., 2000; Hum *et al*., 2000; Sickles *et al*., 2000). **The first outbreak of bovine mastitis caused by mycoplasma in Portugal was diagnosed in 2005 (Pinho *et al*. 2006)**

Bovine mastitis due to *Mycoplasma* is a highly contagious disease that causes elevated economical loses and leads to culling of the infected animals (Counter, 1978; González *et al*., 1990; González, 1996; Wilson *et al*., 1997). This problem is often non identified and can be mantained in the dairies resulting in the contamination of neighbour dairies (González, 1996). Being opportunistic agents, Mycoplasma can colonize quarters that had been previously infected by other organisms (Bushnell, 1984) or increase the rate of mastitis caused by other pathogens or environmental microganisms (Bayoumi *et al.*, 1988; Brown *et al.*, 1990). In effect, an outbreak of mastitis due to Mycoplasma can be missed when more common agents are isolated from clinical mastitis milk (Bushnell, 1984; Judge, 1997). Moreover, Mycoplasma mastitis must be thought when there is a history of chronic mastitis that resist treatment and

when the normal bacteriological results are negative (Tyler & Cullor, 2002).

IV.3 Antimicrobial usage

Antibiotic usage on dairy farms has been blamed for the emergence of antibiotic resistance in humans pathogens (White *et al*., 2001). Despite decades of therapeutic and prophylactic usage, there is no evidence that antimicrobial resistance of mastitis pathogens is increasing in a consistent manner (Markovec & Ruegg, 2002), maybe because the vast majority of dairy farms use antibiotics in a responsible fashion (Ruegg, 2003). The recent Portuguese legislation (Decree-Law 175/2005), will certainly be a significant contribuition for a correct antimicrobial usage.

The *Listeria* genus was thought to be uniformly susceptible to antibiotics, including ampicillin or penicillin (combined with aminoglycosides), trimethoprim (alone or combined with sulfamethoxazole), tetracyclines, erythromycin and gentamicin (Teuber, 1999), active against Gram-positive bacteria, but the first *L. monocytogenes* strains resistant to antimicrobials were detected in 1988 and an increasing number of strains resistant to one or more antibiotics have been reported (Srinivasan *et al*., 2005). *L. monocytogenes* exhibits intrinsic resistance to third-generation cephalosporins, which are therefore incorporated into selective media for its isolation. Antibiotic resistance in *Listeria* species is due to acquisition of movable genetic elements like self-transmissible plasmids and conjugative transposons (Teuber, 1999).

IV.4 Summary

While most aspects of the five-point mastitis control plan have been widely adopted, many other best management practices are not widely used. Routine recording of illnesses and treatments, written standard operating procedures, routine surveillance programs that involve repeated diagnostic tests (such as microbiological testing of bulk tank milk) and participation in quality assurance programs have not been widely adopted across the dairy industry (Ruegg, 2001). Compelling reasons to adopt these practices, such as obvious financial rewards, regulatory oversight or recognizable efficacy and benefit to the farmer, are lacking. Adoption of practices to ensure food safety will face similar challenges (Ruegg, 2003).

V. LISTERIOSIS IN ANIMALS

In this chapter, the main topics about "Listeriosis in Animals" will be reviewed. First the ecology and transmission of *L. monocytogenes* in the farm environment, then pathways and means that *Listeria* use once inside the animal body, and finally Listeriosis clinical evolution, signs and treatments.

V.1 Epidemiology

Virtually all domestic animals are susceptible to listeriosis (Wesley, 1999), with a large proportion of healthy asymptomatic animals shedding *L. monocytogenes* in their faeces, as as already been said. Normal healthy cattle may intermittently shed *Listeria* in their faeces, with prevalence rates ranging from a few percent to 52%, with some seasonality. Faecal shedding may reflect levels of *L. monocytogenes* in feed (Wesley, 1999).

Since listeriosis is not a reportable disease in animals, the exact incidence of listerial infections in domestic livestock remains unknown.

V.2 Ecology and Transmission

L. monocytogenes may enter a herd through contaminated feeds, introduction of new stocks, and rodents. Rodents are known carriers of *L. monocytogenes,* and faecal contamination (fig. 9) of animal feed is a potential source of contamination (Amstutz, 1980).

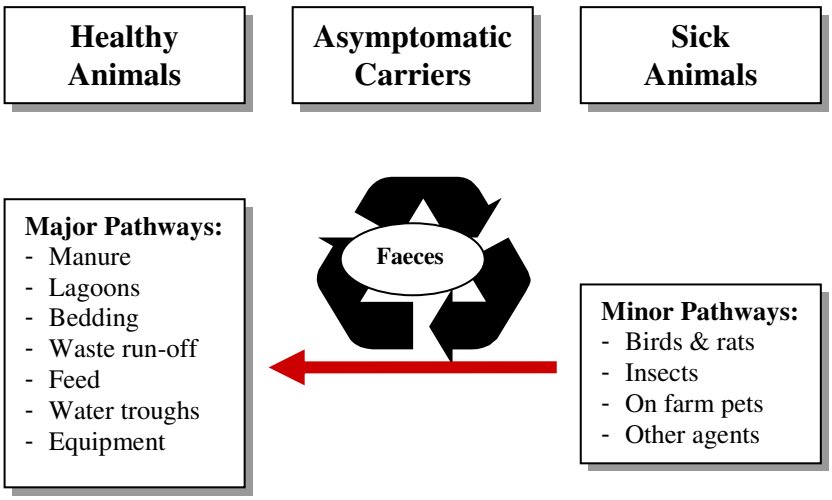


Fig. 9: Maintenance and recycling of foodborne pathogens on the dairy farm. Foodborne pathogens originate from direct contact with contaminated sources, primarily faeces, in the dairy farm environment. The primary source of foodborne pathogens in milk appears to be directly linked to faecal contamination that occurs during the milk processing (adapted from Oliver *et al*., 2005).

According to Wiedmann (2003), in a simplified model (see fig. 10), transmission patterns of *L. monocytogenes* as well as of other foodborne pathogens may encompass all or some of the following steps and environments:

1) bacterial survival in the environment and in animal feeds

2) bacterial survival inside invertebrate hosts (e.g. protozoans)

3) establishment of clinical or subclinical infections or carrier states in food animals

4) shedding of the organism into animal products used for human consumption or secondary contamination of animal products

5) bacterial survival and/or multiplication in non-host environments under food processing and distribution conditions, and

6) infection of human hosts, including survival of gastric passage and establishment of enteric or systemic infection.

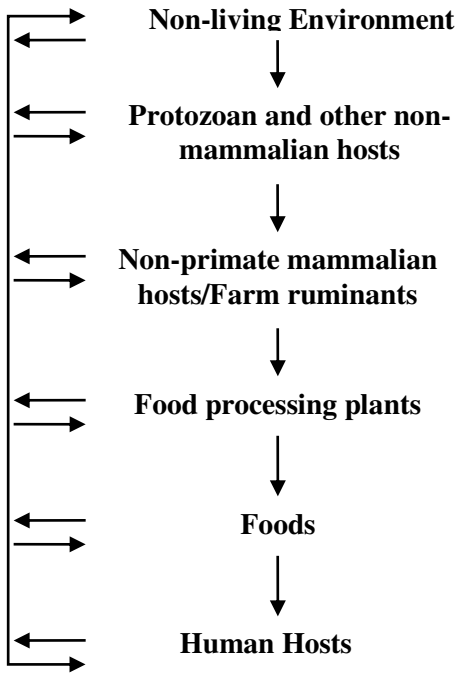


Fig. 10: Transmission pathway model for *L. monocytogenes* (adapted from Wiedmann, 2003)

Data reported by Nightingale *et al*. (2004) support the model (see fig. 11) in which the presence of the pathogen depends on the ingestion of contaminated feed followed by **amplification** in bovine hosts and faecal dissemination in the farm environment. Colonization of the gastro-intestinal tract of the bovine and amplification of *L. monocytogenes* appears to be a required stage in the cell cycle of this foodborne pathogen. Shedding of foodborne pathogens in faeces and distribuition in the environment where cows live assures animal re-infection and persistence of the pathogen on the farm. This, together with the infection of other warm-blooded mammals, birds and insects that live on the farm, place these production units as major reservoirs for foodborne pathogens.

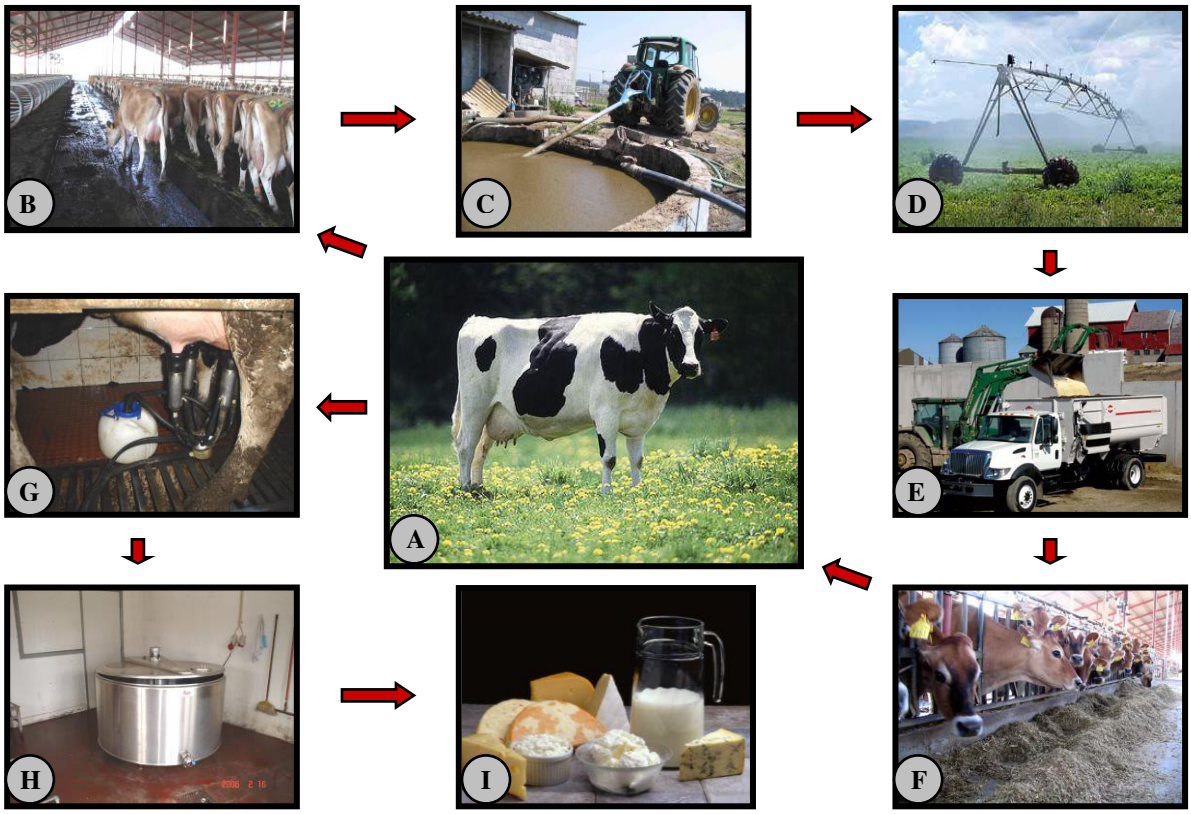


Fig. 11: Cycling of foodborne and veterinary pathogens in the dairy farm environment and their transfer to milk. **(A)** Amplification of the pathogen in the cow. **(B)** Dissemination in the immediate environment of the cow via faeces. **(C)** Accumulation of faeces on the dairy farm. **(D)** Spreading cow manure onto croplands. **(E)** Crops become contaminated with pathogens. **(F)** Contaminated feed consumed by cows. **(G)** Milk can become contaminated with pathogens during milking. **(H)** Pathogens enter bulk tank milk. **(I)** Unpasteurized dairy products made from unpasteurized milk consumed by humans (adapted from Oliver *et al*., 2005).

Foodborne transmission is the main **mode of infection** in naturally occurring listeriosis in cattle with silage (fig.12) being most frequently implicated (Muraoka *et al*., 2003). Poorly-ensiled silage, with pH values higher than 4.5 (Gonzalo *et al*., 2004), can contain more than 10^7 cells/g (Bemrah *et al*., 1998). Bovine abortions and stillbirths occur shortly after contaminated silage is fed (Amstutz, 1980).

Fig. 12: Silage.

In addition, animal listeriosis cases sometimes occur in animals that are not fed silage and environmental sources have been speculated to be responsible for at least some of these cases. In fact, since *L. monocytogenes* is present in soil, faecal material, and vegetation, it may enter via abrasions of the nostrils or the conjunctiva while grazing or via the teat of a lactating cow. Direct injection of the conjunctiva, resulting in keratoconjunctivitis, has occurred as a result of contaminated silage particles falling into the faces of browsing cattle (Wesley, 1999). The agricultural environment thus may serve not only as an important source for contamination of silage, but may also be a direct source of animal infection in some cases (Wiedmann, 2003).

V.4 Dissemination pathway

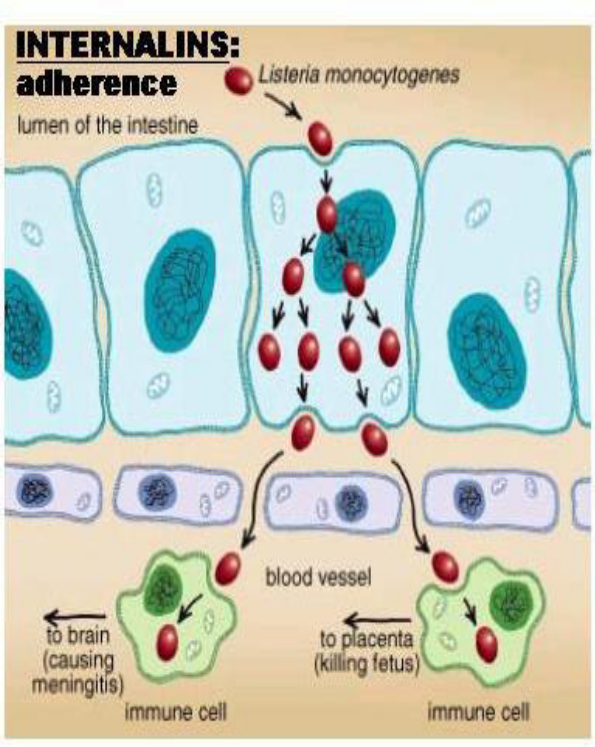


Fig. 13: Dissemination pathway. *in* http://web.indstate.edu

Listeriae belong to a group of bacteria that are able to penetrate into, and to survive and multiply within host cells, not only in professional phagocytes but also in virtually every nucleated cell of the body. By means of factors such as internalin A and/or B the host cells are triggered to internalize the attached bacteria (fig. 13). Inside a host cell the pathogenic bacterium produces hemolysin and phospholipases and it can

leave the phagocytic vacuole and enter the cytoplasm. Once in the cytosol, pathogenic strains will polymerize actin from the host cell cytoskeleton. This happens mainly at the apical tip of a bacterium so that the new actin filaments act like a driving force; the bacterium moves around in the host cell until it by chance gets in contact with the cell membrane of the host cell. This contact induces the host cell membrane to produce extrusions which penetrate a neighbouring host cell. The second host cell can engulf these ramifications containing the living *L. monocytogenes*. Hence, the bacterium will lyse the double cell membrane and invade the cytoplasm of the second host cell. By this mechanism of cell-to-cell spreading the intracellular parasite avoids the host cell´s defense mechanisms (Hof, 2003).

V. 5 Clinical signs

Although most infections are subclinical, listeriosis in animals can occur either sporadically or as epidemics and often leads to fatal forms of encephalitis. Besides encephalitis, the main symptoms of bovine listeriosis include abortion and septicaemia with miliary abscesses.

Following ingestion, *Listeria* is disseminated via hematogenous spread to the viscera, brain and gravid uterus. By travel along peripheral nerves (indicated by Roman numerals), especially the hypoglossal (XII) and trigeminal (V) cranial nerves innervating the buccal cavity, *L. monocytogenes* enters the central nervous system and localizes in the pons and medulla. Damage to the cranial nerves underlies the clinical presentation. For example, lesions of the fifth (V) cranial and mandibular nerves lead to inability to eat or drink or to retain food in the mouth. Excessive salivation from difficulty in swallowing (IX and X) and protrusion of the tongue (XII); ataxia or circling (VIII); facial paralysis, including unilateral drooping of the lip, ear (fig. 14), and eyelid (VII); and strabismus (VI) reflect damage to the respective cranial nerves (Rebhun, 1987).

Fig. 14: Calf showing typical signs of listeriosis, like unilateral drooping of the ear.

In the advanced stage, as vision and locomotion are impared and the animal becomes increasingly irritable, the illness may be confused with rabies or lead poisoning. Finally, the animal lapses into a coma and generally dies within 1-2 days (Wesley, 1999). However, even in acute outbreaks, generally no more than 8-10% of a herd succumbs to infection.

Histopathological lesions of the brain stem consist of foci of necrosis infiltrated with neutrophils, macrophages, and bacteria. Perivascular cuffing with mononuclear cells is evident (Timoney *et al*., 1988). Unlike listerial encephalitis in sheep and goats, most cattle survive at least 4-14 days after the initial onset of symptoms, with a few reports of spontaneous recovery.

Listeriosis in cattle is frequently associated with abortion, which generally occurs during the last trimester of pregnancy. However, healthy calves can be born to chronic carriers that shed the pathogen in milk (Wesley, 1999). As in sheep, *L. monocytogenes* is transmitted to the placenta, and then into the fetus. Meningitis in neonates may follow intrauterine infection with the septicemic young animal dying shortly after birth.

V.6 Treatment

Poor animal husbandry, consumption of contaminated feed, and stress are important factors in precipating listeriosis. Thus identifying and eliminating these problems are critical to preventing re-occurrences. In general, since antemortem diagnosis is rarely made, treatment is seldom attempted.

Since listerial encephalitis is a rapidly debilitating disease in ruminants, treatment must be initiated early during the course of infection if there is to be any reasonable hope of a cure. The intravenous injection of chlortetracycline (10 mg/kg body weight per day

for 5 days) is usually effective in treating meningoencephalitis of cattle (Wesley, 1999). If penicillin is used, high doses are required because of the difficulty of maintaining therapeutic levels in the brain. Penicillin G should be given at 44,000U/Kg body weight, intramuscularly daily for 1-2 weeks (Fraser *et al.*, 1991). If signs of encephalitis are severe, death usually occurs in spite of treatment. Supportive therapy, which is usually reserved for valuable animals, including fluid and electrolyte replacement, is indicated for animals having difficulty eating and drinking as a result of neural damage. Excessive salivation leads to acidosis, which is remedied by intravenous replacement of bicarbonate ions. Permanent neurological damage often occurs in ruminants despite proper therapy.

Stress-related immunosuppression associated with change of diet, weather, transport, pregnancy, parturition and lactation, may lower resistance to bovine listeriosis. Dexamethasone mimics the stress-related release of glucocorticoids. In cattle, dexamethasone elevates total white blood neutrophil counts and decreases eosinophil and lymphocyte populations. When administrated to cows experimentally infected with *L. monocytogenes*, dexamethasone increased shedding of the pathogen in milk by up to 100-fold (Wesley *et al*., 1989).

Prompt treatment of animals with listeriosis is clearly beneficial, with early diagnosis dependent on observation of clinical symptoms. In cattle and sheep, appearance of clinical signs is an indication of neurological damage and thus, of a guarded prognosis for treatment (Wesley, 1999).

VI. LISTERIOSIS IN HUMANS

In this chapter Listeriosis (in Humans) epidemiology, clinical manifestations (with particular attention to the unique complications during pregnancy), diagnosis and treatment will be reviewed.

VI.1 Epidemiology

Human listeriosis has a world-wide distribution, and in the USA, although less common than many other foodborne diseases, represents the second most frequently identified cause of death from a foodborne illness (Guerra *et al*., 2001)(the **mortality rate** is approximately 25%, but may be as high as 50% in the neonatal population (DiMaio, 2000)), next only to *Salmonella* infections, and is associated with the highest hospitalization risk (Pak *et al*., 2002). Listeriosis occurs at a rate between 4.4-7.4 per million population annually (Lorber, 1997).

The bacterium has been recovered from approximately 5-15% of normal adult stool samples (i.e., healthy people can be carriers of *L. monocytogenes* (Salo *et al*., 2006)), and from up to 25% of samples from household contacts of patients who are clinically ill (Rocourt & Bille, 1997). The vagina, cervix and pharynx are other sites for potential carriage of the organism (DiMaio, 2000).

The rates of infection are highest at ages less than one month and greater than 60 years. Death attributed to listeriosis is rare in patients less than 65 years of age without a predisposing condition. Death occurs, on the average, within 30 days with sepsis and within 40 days for central nervous system (CNS) disease. Pregnant women constitute between 20-30% of all cases and 60% of all cases in the reproductive age group (Lorber, 1997). However, despite the predilection for pregnant women, listeriosis occurs in men almost twice as often as women, since men are more likely to harbor underlying conditions (Goulet & Marchetti, 1996). Forty to 70% of cases in the non-pregnant population are found in those with Acquired Imuno Deficiency Syndrome (AIDS), malignancies (especially leukaemia and lymphoma), organs transplants, those suffering from alcoholism, and those receiving long therm corticosteroid treatment. Patients without underlying medical conditions account for less than 20% of cases (Goulet & Marchetti, 1996). Most cases of listeriosis are sporadic in nature, but there have been

several reports implicating a common food source outbreak, as refered in section III.3.2, table 6.

VI.2 Infectious dose and incubation period

The **infectious dose** cannot be stated with precision (Jensen *et al*., 1996): small doses may infect immunocompromised hosts but much larger doses are required for normal individuals; according to Holko *et al*. (2002) it is 1000 per g. Baumgart (1993) reported that the cheese contained 10^3-10^4 *L. monocytogenes* per gram caused to food borne illness.

The **incubation period** is approximately 2-6 weeks (Fauci *et al.*, 1998). H2 blockers, antacids, laxatives and ulcer surgery have all been shown to promote disease, indicating that gastric acid has a protective effect against infection (Goulet & Marchetti, 1996; Lorber, 1997).

There have been no documental cases of human to human transmission of *Listeria,* although there have been clusters of infections in veterinarians and those who come in close contact with animals. *L. monocytogenes* can infect humans and other animals by oral, ocular, cutaneous, respiratory or urogenital routes (Pearson & Marth, 1990). There have been also clusters of late neonatal infection, suggesting the possibility of nosocomial transmission (DiMaio, 2000).

Once the organism penetrates the mucosal barrier of the intestine, it spreads hematogenously, most often to the CNS or placenta. Invasive listeriosis may occur more readily if there is a coinfection with another pathogenic organism such as *Salmonella* or *Shigella* (DiMaio, 2000). *L. monocytogenes* is more likely to gain access to the blood stream after a procedure that may breech the mucosal barrier, such as colonoscopy or bowel surgery (Lorber, 1997). Iron also seems to be an important promoter of virulence. Iron increases the growth of the organism in vitro and states of iron overload, such as hemochromatosis, predispose the affected patient to listeriosis (Lorber, 1997).

VI.3 Clinical manifestations

There is scant evidence that a mild and transient gastroenteritis may precede overt disease. Obviously, this exposure to pathogenic *Listeriae* is rather common, since more than 90% of adults possess immune lymphocytes. Whereas most normal,

immunocompetent individuals, will overcome an initial attack and shedding of *Listeriae* by faeces is terminated after a few days, people at risk may suffer from disseminated infection (Hof, 2003), with the following possible clinical manifestations:

3.1 Sepsis

Sepsis without a localized infection is the most common presentation in patients with deficient immune systems. The patient often appears severely ill, with fever, nausea, vomiting and malaise. Sepsis may progress to disseminated intravascular coagulation (DIC), adult respiratory distress syndrome (ARDS) and multi-organ system failure. This spectrum of disease is often identical to other types of bacterial disease and requires a positive blood culture to establish a diagnosis (Lorber, 1997; Fauci *et al.*, 1998).

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3.2 CNS infection

CNS disease is the second most common presentation of *L. monocytogenes* infection in the immunocompromised population, and the most likely presentation of listeriosis in the healthy population. The most frequent manifestation of infection is **meningitis** (Goulet & Marchetti, 1996), and, in fact, *L. monocytogenes* is among the five most common causes of bacterial meningitis (DiMaio, 2000). Meningitis secondary to *Listeria* presents similarly to other forms of meningitis and is, likewise, often devastating. The clinician should be highly suspicious of this organism if the patient is immunosuppressed (Lorber, 1997; Fauci *et al.*, 1998).

L. monocytogenes is distinguished from the common meningitis pathogens (*Streptococcus agalactiae*, *Haemophilus influenzae*, and *Neisseria meningiditis*) in that it has a special predilection for brain parenchyma (DiMaio, 2000). Patients with this type of meningitis are often found to have coexisting cerebritis and brain abcesses (Fauci *et al.*, 1998). The organism also shows preference for the brainstem and causes a disease similar to circling disease found in sheep, called rhomboencephalitis in humans (DiMaio, 2000). Rhomboencephalitis is usually exhibited by 3-5 days of non-specific fever, nausea, vomiting and headache (Fauci *et al.*, 1998). Coma may be the initial symptom in up to 30% of patients and is seen most often in the older and more immunosuppressed population (Goulet & Marchetti, 1996).

After this first stage of disease, the patient will have the onset of hemiparesis, altered level of consciousness, sensory loss, cerebellar signs and asymmetrical cranial nerve deficits. Close to 50% of patients also develop respiratory distress and failure. **Nuchal rigidity** is only present 50% of the time, and Cerebral Spinal Fluid (CSF) cultures may be sterile in 60% of specimens (DiMaio, 2000). Analysis of the CSF may show a "false-negative" Gram stain, pleocytosis, increased protein and normal glucose concentration. The normal glucose concentration allows listeriosis to be differentiated from other types of bacterial meningitis (DiMaio, 2000). In 60-75% of cases, blood cultures are positive (Lorber, 1997). The course of rhomboencephalitis is usually so severe that patients either die or have serious residual neurological disease. Recurrence is rare (Lorber, 1997).

Listeria may also present with brain abcesses in about 10% of cases when the CNS is involved (Lorber, 1997; Fauci *et al.*, 1998). Abcesses are particulary likely to occur in the immunosuppressed population, and the subsequent mortality rate is quite high. 25% of patients also have meningitis, and almost all patients become bacteremic. *Listeria* also uniquely forms abcesses in subcortical areas such as the medulla, pons and thalamus (DiMaio, 2000).

3.3 Endocarditis

Listerial endocarditis is responsible for 8-10% of all listerial infections (DiMaio, 2000). This manifestation is usually found in those with a prosthetic cardiac valve or those who have previously damaged and scarred valves. However, the organism has also been reported in native valve infections. *Listeria* has been found to preferentially infect left-sided valves and is often a source of systemic bacterial emboli (Fauci *et al.*, 1998). The mortality rate for this infection is approximately 50% (Lorber, 1997). Patients with listerial endocarditis should be examined for an underlying gastrointestinal cancer because the two have been found to be associated (DiMaio, 2000).

3.4 Gastrointestinal disease

In a healthy population, consumption of foods contaminated with *L. monocytogenes* may cause a self limited syndrome presenting with fever, nausea, vomiting and diarrhea (DiMaio, 2000). This disease should be considered when stool

cultures are negative in a patient with acute gastroenteritis. Patients presenting with CNS or cardiac manifestations often report preceding gastrointestinal symptoms (Lorber, 1997).

3.5 Focal infections

Listeria not only causes systemic disease, but also localized infections such as cellulitis and conjunctivitis (DiMaio, 2000). These superficial infections are most commonly found in veterinarians and other animal workers (Rocourt & Bille, 1997; Fauci *et al.*, 1998). *Listeria* bacteremia has been implicated in a number of diseases and has been a cause of peritonitis, cholecystitis, hepatitis, pleuritis, splenic abscess, pericarditis, osteomyelitis, endophthalmitis (Fauci *et al.*, 1998), pneumonia and urethritis (Pearson & Marth, 1990). The above infections may be the result of septic emboli with listerial endocarditis. These infections are most commonly seen in patients who are immunocompromised (DiMaio, 2000).

VI.4 Unique complications in Pregnancy

Cell-mediated immunity is slighty decreased during pregnancy, and this alteration places the pregnant woman at risk for listerial disease (DiMaio, 2000). According to Hof (2003) pregnant women have a 12-fold increased risk in comparison with the normal population to acquire listeriosis after consumption of contaminated food, and so it seems advisable for this specific risk group to change its food habits during pregnancy. In a survey by Paula Teixeira (unpublished data), of 312 women, only 54% changed their food habits during pregnancy.

Listeriosis is an unlikely cause of habitual abortion, although it often is included in the differential diagnosis (Lallemand *et al*., 1992). The infection is most commonly seen during the third trimester, which may be secondary to a further decrease in immune system function. However, the disease may occur at all stages of gestation (DiMaio, 2000). *L. monocytogenes* has a predilection for the placenta, which is often unreachable by the immune system. It is interesting to note that, in listeriosis during pregnancy, CNS infection is rarely seen in the absence of other pre-existing risk factors. The disease usually presents with bacteremia, and the most common manifestations are fever (often greater than 39 ºC), headache, myalgia, arthralgia, and malaise (DiMaio, 2000). The

bacteremia often results in hematogenous spread and transplacental infection which, in turn, may lead to chorioamnionitis, premature labor, premature rupture of membranes, intrauterine fetal demise, or early-onset infection in the neonate (Lorber, 1997). Signs of intrauterine infection include diarrhea, nausea, vomiting, backaches, abdominal pain, and bloody vaginal discharge (Kalstone, 1991). Ascending infection through intact membranes is uncommon, but infection during passage of the fetus through the vagina may occur. In addition, nosocomial transmission may occur in the newborn nursery (DiMaio, 2000).

If a mother becomes infected with *L. monocytogenes* the fetus is affected in more than 90% of cases (DiMaio, 2000). Up to 22% of cases of listeriosis result in stillbirth or neonatal death. Infection that occurs early in pregnancy is more likely to result in fetal death; after 20 weeks´ gestation infection is more likely to result in preterm labor (Topalovski *et al.*, 1993). The amniotic fluid of affected women is more likely to be meconium-stained, even at an early gestational age, probably secondary to ingestion of infected fluid resulting in enteritis and passage of fetal waste products (DiMaio, 2000). Women who are treated promptly with antibiotics during pregnancy, or who are delivered, usually have decreased morbidity and mortality, a fact which highlights the importance of making an early and accurate diagnosis of listeriosis (DiMaio, 2000). In a pregnant woman who presents with fever and preterm labor, blood, cervical and amniotic fluid cultures should be obtained (Lorber, 1997).

The placenta is often found to have gross abscesses by visual inspection, as well as microabscess with necrosis by microscopy. The abscesses are usually multiple, well circumscribed, gray and solid (DiMaio, 2000). The appearance is often confused with infarction of the placenta; thus the placenta always should be sent for microscopic evaluation when listeriosis is suspected. Organisms can easily be identified with silver impregnation stains, even if the Gram stain is not conclusive (Topalovski *et al.*, 1993) .

VI.5 Neonatal infection

Listeria infection of the fetus may immediately result in spontaneous abortion or stillbirth. Listeriosis should be suspected when there is a spontaneous abortion and a fever preceded the fetal loss by 24-48 hours (Lallemand *et al.,* 1992*)*. However, if the fetus suvives, neonatal listeriosis has a similar course to group B streptococcal infection.

The spectrum of disease can be divided into **early** and **late onset** listeriosis:

- Early-onset disease occurs within one week of delivery but usually manifests within two days postpartum. Early-onset disease is probably acquired in utero and often presents in the preterm infant with sepsis, respiratory distress, purulent conjunctivitis and skin lesions (Lorber, 1997). The highest inoculum of bacteria is usually found in the lung and gut, implicating aspiration of infected amniotic fluid by the fetus in utero as a mode of infection (DiMaio, 2000). A particularly devastating form of early onset listeriosis is called *granulomatosis infantiseptica*, and it is characterized by widespread abscesses and granulomas on the skin and in the visceral organs of the neonate (Fauci *et al.*, 1998). The infants are often stillborn or expire in the immediate puerperium, and there is typically a high concentration of bacteria in the meconium of the neonate (DiMaio, 2000).

- Late-onset disease is usually diagnosed 1-2 weeks postpartum and is most commonly found in full-term infants. The infants usually have uncomplicated deliveries, and the mode of transmission is probably during passage through the birth canal (DiMaio, 2000). It is interesting to note that late-onset disease has been seen in infants delivered by cesarean delivery, which is evidence supporting nosocomial infection (Lorber, 1997). Those who present with late-onset disease are more likely to have meningitis than those with early-onset infection. The complications of perinatal infection are often grave but can be ameliorated with prompt recognition and treatment during pregnancy (DiMaio, 2000).

VI.6 Diagnosis

It is imperative that the clinician maintain a high index of suspicion for *L. monocytogenes* infection. The health care provider must remain wary in the case of CNS symptoms in an immunocompromised patient, meningitis and parenchymal brain infection in the same patient, fever in the pregnant woman, neonatal sepsis or meningitis, and food-borne or febrile gastroenteritis where routine cultures are without growth. Diagnosis requires growth of the organism from bodily fluids that are normally considered sterile (DiMaio, 2000). The clinician should obtain blood, amniotic fluid and

CSF for culture if listeriosis is suspected (Lorber, 1997). CNS involvement may often be diagnosed and followed by magnetic resonance imaging (MRI). The bacteria will grow easily on routine culture medium within 24-36 hours; however, the possible confusion with diphtheroids and *Streptococci* make biochemical testing important in identification of the species. Newly adapted tests with antibodies to listeriolysin O may assist in confirming the infection (DiMaio, 2000).

VI.7 Treatment

Ampicillin is the treatment of choice for listeriosis, in humans (DiMaio, 2000). Interestingly, β-lactam antibiotics are only bacteriostatic rather than bacteriocidal for *Listeria* (DiMaio, 2000). In a patient with listerial meningitis or endocarditis, or in a severily immunocompromised patient, the addition of gentamicin is indicated (Fauci *et al.*, 1998). In the penicillin-allergic patient, trimethoprim-sulfamethoxazole is effective in eradicating the disease. All patients should be treated with doses high enough to penetrate the CNS, regardless of wether the patient has obvious signs of CNS involvement, because of the high affinity of *L. monocytogenes* for these tissues. Corticosteroids, which are often used in the treatment of other types of bacterial meningitis, should be avoided in listerial meningitis, for fear of causing a further decrease in immune competency (Lorber, 1997). During pregnancy, prompt treatment can significantly decrease the rate of fetal infection and morbidity and mortality in the neonate. Ampicillin achieves a therapeutic blood level in the fetus in utero (DiMaio, 2000). Prenatal vitamins or iron supplementation should be temporarily discontinued because iron appears to enhance the virulence of the organism (Lorber, 1997).

VII. MATERIAL AND METHODS

In this chapter, a description of the field and laboratory work (see fig. 16) that was done integrated in this MS project, will be done.

VII.1 Samples collection

The field study took place between **December 2005 and June 2006**. **166 raw milk samples** (aliquots of approximately 80 mL), from **39** different **dairy farms** from **13** different Northern Portuguese coast localities, were collected into sterile containers (Table 8) using aseptic techniques. They were then transported to the laboratory in cold, insulated containers, containing ice refrigerants, and stored at 4 ºC until analysis, normally until 7-10 days after collection.

Of the 166 raw milk samples, 45 (27.11%) were from healthy cows (composite samples of the 4 teats), 58 (34.93%) were collected from cows presenting sub-clinical mastitis (according to the results obtained by the Californian Mastitis Test- CMT; Laboratório Sorológico, Portugal, fig. 15); 27 (16.27%) were from cows presenting clinical mastitis (milk was macroscopically abnormal) and 36 (21.69%) were bulk tank samples.

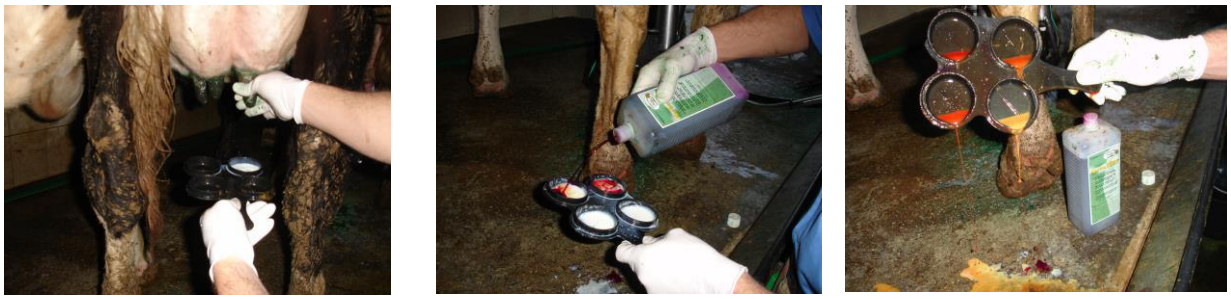

Fig. 15: Californian Mastitis Test: Equal amounts of milk and test reagent are mixed (left and midle figs.). If the mixture remains fluid, SCC is probably below 350,000, i.e., for a naked eye there is no sub clinical mastitis. In the presence of a higher SCC, a viscous gel is formed.

As it can be observed in Table 8, 3 of the sub-clinical mastitis, 9 of the bulk tank and 2 of the healthy cows milk samples were analysed twice; before freezing and after freezing and subsequent defrosting.

Table 8: Milk samples collected.

Localities	Dairy Farm number	Milk from			
		Healthy Cow	Sub Clinical Mastitis	Clinical Mastitis	Bulk tank
Aveiro	12	0	0	0	1
	14	0	0	0	1
Barcelos	1	0	5	2	0
	5	0	13	6	0
	6	0	0	0	1
	7	0	0	0	1
	9	0	0	0	1
	10	0	0	0	1
	17	8	2	1	1(2x)+1
	19	0	0	0	1(2x)
	28	0	0	0	1
	32	0	0	0	1
Estarreja	11	0	0	0	1
	22	0	0	0	1(2x)
	24	0	0	0	1(2x)
	25	0	0	0	1(2x)
Maia	33	6	0	1	0
	34	0	0	2	0
	36	4	1	0	0
	38	0	0	0	1
Matosinhos	8	0	0	0	1
	18	0	0	0	1(2x)
Ovar	13	0	0	0	1
Paços Ferreira	37	0	0	0	1
Ponte Lima	31	0	0	0	1
Póvoa Varzim	30	0	0	0	1
Santo Tirso	15	4	3	0	0
Trofa	26	0	2	1	0
Vila Conde	3	8	6	3	0
	4	5	1(2x)+3	2	0
	16	0	0	0	1(2x)
	21	0	0	0	1(2x)
	23	0	0	0	1(2x)
	20	4	0	0	0
	27	1	8	0	0
	29	0	0	0	1
	39	0	0	1	0
V.N. Famalicão	2	2(2x)	2(2x)+9	8	1
	35	1	0	0	0
TOTAL: 13	39	45	58	27	36

Legend: 2x means that twice the volume was collected in these cases; Half of it was analyzed fresh and the rest after freezing and subsequent defrosting.

From farm 17 (in Barcelos), a sample of silage, *unifeed* (a totally mixed ration of all the components of a dairy cow daily diet), cattle manure and drinking water were analysed for *L. monocytogenes* detection and enumeration, as well as environmental samples from: floor (milk parlour and barn), bulk tank, buckets (for the teat cleaning towels and liners), footbath, walls and liners.

VII.2. Detection and enumeration of *L. monocytogenes*

The detection was performed using the VIDAS technology (anonymus, 1996) and enumeration of *L. monocytogenes* was performed based on the International Organization for Standardization (ISO) standard procedure 11290-2 (anonymous, 1998) and on the five-tube most probable number (MPN) technique for enumerating bacterial pathogens as described by the US Food and Drug Administration (FDA; anonymous, 1992). The media used for the MPN technique were those detailed in the ISO standard procedure 11290-1 (anonymous, 1996) for *L. monocytogenes* detection.

VII. 2.1 Detection - VIDAS methodology

The VIDAS LMO assay is an enzyme-linked fluorescent immunoassay (ELFA) for use on the VIDAS system for the qualitative detection of *L. monocytogenes.* The internal surface of the Solid Phase Receptacle (SPR), a pipette tip-like disposable device, is pre-coated during production with anti-*L. monocytogenes* antibodies. The VIDAS LMO assay configuration prevents non-specific reactions with the SPR. Reagents for each assay are held in a sealed multi-chambered strip.

The VIDAS instrument performs all assay steps automatically and sequentially. Each sample is inoculated into the reagent strip and cycled in and out of the SPR for a specific length of time. *L. monocytogenes* antigens present in the sample will bind to the anti-*L. monocytogenes* monoclonal antibodies, which are coated on the interior of the SPR. Unbound sample material is washed away. Antibodies conjugated with alkaline phosphatase are then cycled in and out of the SPR and react with the *L. monocytogenes* antigen-antibody complexes already adsorbed to the SPR wall. Unbound conjugate is removed by washing. The fluorescent substrate, 4-methyl-umbelliferyl-phosphate, is

then cycled in and out of the SPR where the enzyme conjugate converts the substrate to fluorescent 4-methyl-umbelliferone. The intensity of fluorescence is then measured at 450 nm.

In this study, milk (and water) samples (25 mL) were placed in 225 mL half-Fraser broth (Biokar Diagnostics, Beauvais, France), homogenized for 2 min, and incubated at 30 ºC for 24 hours. For silage, *unifeed,* and cattle manure samples, 25 g were used. They were mixed with 225 mL of half-Fraser with the help of a Stomacher (BagMixer 400, Interscience, St Nom, France). Environmental samples were taken with the help of sterile cotton swabs, which were then placed into sterile tubes with 10 mL of half-Fraser broth, and also incubated at 30 ºC, for 24 hours.

Aliquots (0,1 mL) of these primary enrichments were transferred to 10 mL of secondary enrichment, Fraser broth (Biokar), and incubated at 30 ºC for 24 hours. The sample wells of VIDAS LMO reagent strips (BioMérieux, SA, Marcy l´Etoile, France) were inoculated with 0.5 mL of each secondary enrichment broth. The results were obtained automatically after 70 minutes. Enrichment broths were stored at 2-8 ºC and, when samples were positive in the VIDAS assay (Test value ≥ 0.05), were streaked on PALCAM (Merck, Darmstadt, Germany) and ALOA (BioRad Laboratories, Amadora, Portugal), and incubated at 37 ºC for 48 hours. Five typical colonies per plate (when possible) were selected for confirmation according to the ISO guidelines, as it will be described in section VII.3.

VII.2.2 Enumeration

VII.2.2.1 Most probable number

Milk samples were tested using the five tube MPN technique for enumerating bacterial pathogens in foods as described by the US FDA (Anonymous, 1992). The media used for the MPN technique were those detailed in the standard procedure ISO 11290-1 for *L. monocytogenes* detection.

A 10 mL aliquot of each milk sample was inoculated into each of five tubes with 10 mL of half Fraser selective broth (double concentration). Two additional five tube sets of half Fraser selective broth were inoculated with 1 mL and 0.1 mL of the initial milk samples, respectively. For the water sample, a similar procedure was done. For the silage, *unifeed* and cattle manure sample, aliquots were taken from the VIDAS pre-

enrichment mixture, described in section VII.2.1. After the tubes were incubated at 30 ºC for 24 h, 0.1 mL were transferred from each tube of half Fraser selective broth to a tube containing 10 mL of Fraser selective broth with subsequent incubation at 37 ºC for 48 h. After incubation, from each tube of Fraser selective broth a loop was streaked on PALCAM and ALOA, and were incubated at 37 ºC for 48 h. Five *Listeria*-like colonies on PALCAM and ALOA (when possible) were picked up and were subcultured on tryptone-soy agar plates supplemented with 0.6% yeast extract (TSAYE, Merck & Co., Inc., Whitehouse Station, N.J., USA), a non selective medium, and confirmed as *L. monocytogenes* by standard procedures, described in section VII.3.

The MPN index was calculated from the number of tubes confirmed to be *L. monocytogenes* positive, using the FDA MPN table available online at http://www.cfsan.fda.gov/~ebam/bam-a2.html#excl.

VII.2.2.2 *Direct Enumeration*

500 µL of each milk sample were spread on PALCAM and ALOA agar (two plates of each medium) and further incubated at 37 ºC for 48 h. For the water sample, a similar procedure was performed. For the silage, *unifeed* and manure samples, 500 µL were taken from the VIDAS pre-enrichment mixture, described in section VII.2.1. *L. monocytogenes* suspected colonies were confirmed by standard procedures described in section VII.3.

VII.3. Confirmation of the species *L. monocytogenes*

VII.3.1 Sugars fermentation

One isolated colony, from the subculture on TSAYE, was streaked on a plate of Purple agar (16g/L purple broth base plus 15g/L agar (Becton Dickinson, Sparks, USA)), containing each sugar (all sugars were supplied by Sigma, Steinheim, Germany): mannitol (0.5 % w/v), rhamnose (1 % w/v) and xylose (1 % w/v). Plates were incubated at 37 ºC for 24 h. Positive results (yellow color) were analysed according to table 9.

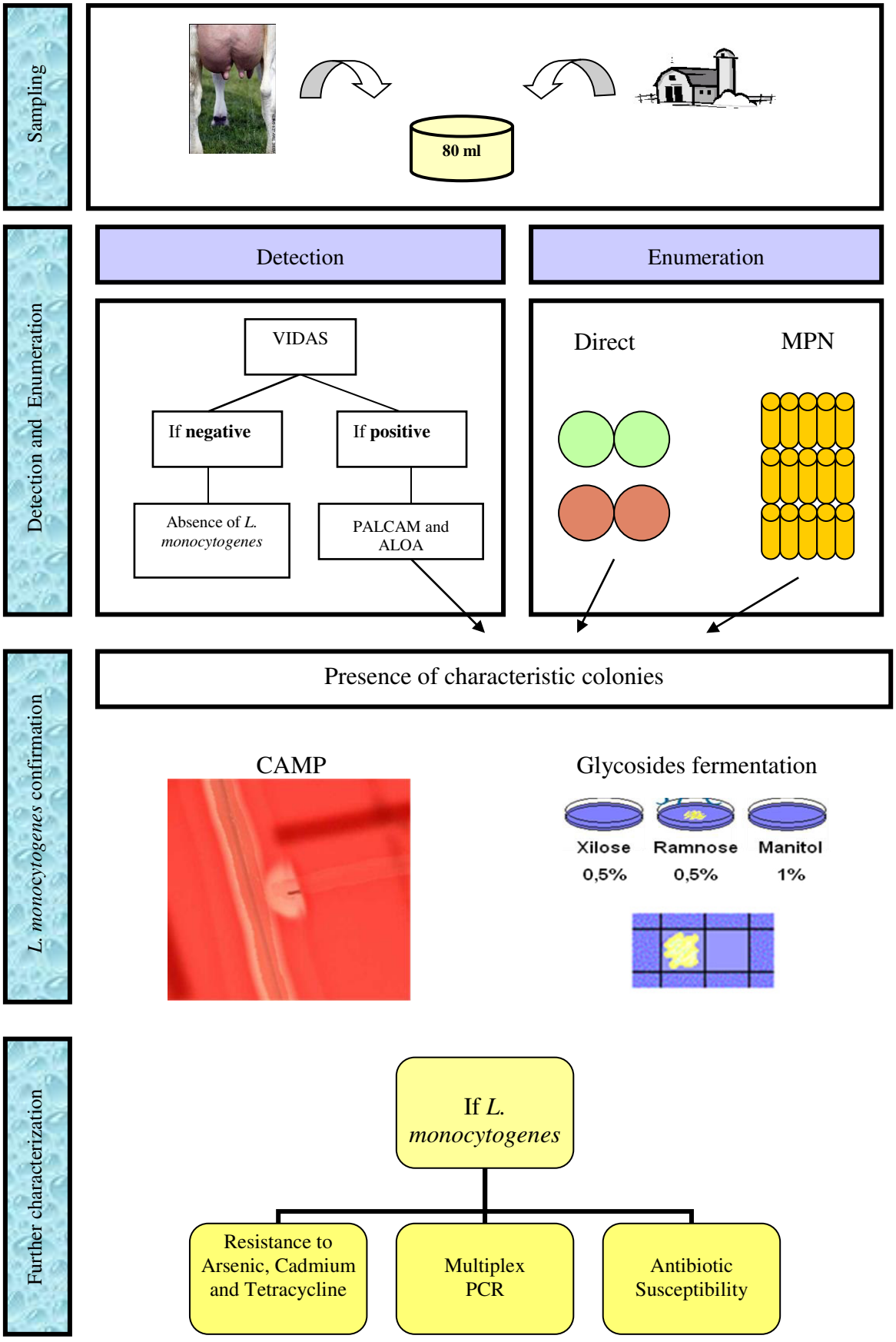

Fig. 16: Schematic representation of the field and laboratory work.

Table 9: Sugars interpretation results (adapted from FDA, 2003).

Species	Sugars		
	Mannitol	Rhamnose	Xylose
L.monocytogenes	-	+	-
L. ivanovii	-	-	+
L. innocua	-	V	-
L. welshimeri	-	V	+
L. seeligeri	-	-	+
L.grayi	+	V	-

Legend: - = negative result; + = positive result; V= Variable biotypes

VII.3.2 Christie, Atkins, Munch, Petersen (CAMP) test

Single-line streaks of *S. aureus* (American Type Culture Collection (ATCC) 4944 or ATCC 25923) and *Rhodococcus equi* (ATCC 6939), reference cultures, were applied on a sheep blood agar plate in parallel and 3-4 cm apart. Test cultures were streaked between and perpendicular to the two reference cultures (i.e. like rungs of a ladder) (fig. 17). The test culture streak was 2-4 mm from each reference culture streak. Plates were incubated 24 h at 37 °C. Test culture streaks were examined for enhanced β-hemolysis at both ends proximal to the reference cultures. The presence of a zone of enhanced β-hemolysis (that may resemble an arrowhead, circle or rectangle) indicates a CAMP-positive reaction (fig. 16). Absence of enhanced β-hemolysis indicates a CAMP-negative reaction, characteristic of *L. innocua* and *L. welshimeri*. *L. monocytogenes* and *L. seeligeri* are CAMP-positive to the *S. aureus* reference strain and CAMP-negative to *R equi* (rare strains of *L. monocytogenes* are positive for both control strains). In contrast, *L. ivanovii* is CAMP-positive to the *R. equi* reference strain and CAMP-negative to the *S. aureus* strain (FDA, 2003).

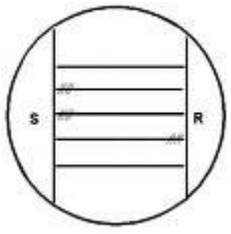

Fig. 17: Scheme of CAMP test.
Legend:S- *S.aureus* R- *R. equi*

For both confirmation tests, *L. monocytogenes* NCTC 11994, from Health Protection Agency (HPA) collection was used as positive control.

VII.4 Characterisation of *L. monocytogenes* isolates

L. monocytogenes isolates were stored at -20 ºC in TSB (Tryptone-Soy Broth) (LabM, Lancashire, United kingdom) plus 30% glycerol (Pronalab, Abrunheira, Portugal) and every time they were needed, they were streaked in TSAYE and incubated for 24 h at 37 ºC. Working cultures were also grown in TSAYE and further characterized as described in sections VII.4.1, VII.4.2 and VII.4.3.

VII.4.1 Resistance to Arsenic, Cadmium and Tetracycline

Cultures that were previously identified as *L. monoctogenes* were inoculated into 5 mL nutrient broth Tryptone-soy broth supplemented with 0.6 % (w/v) yeast extract (TSBYE) and incubated overnight at 37 ºC. Resistance to cadmium and arsenic was determined as described by McLauchlin *et al.* (1997): cultures were homogeneized and 3 µL of broth cultures were inoculated onto Isosensitest agar plates (Oxoid, Basingstoke, UK) containing 500 µg/mL sodium arsenite (Merck Ltd, Poole, Dorset, UK) or 75 µg/mL cadmium chloride monohydrate (Merck). A control isosensitest agar plate was also inoculated. Plates were incubated overnight at 37 ºC and the growth on arsenic-and cadmium-containing agar was compared with that on control.

Resistance to tetracycline was determined using a similar method, described by Vaz-Velho *et al.* (2001): overnight cultures were inoculated onto an Isosensitest agar plate containing 8 µg/mL of tetracycline-HCl (Sigma, Aldrich, Poole, Dorset, UK). Growth was compared with an Isosensitest plate without tetracycline after overnight incubation at 37 ºC.

The following strains were used as controls for the arsenic, cadmium and tetracycline sensitivity: *L. monocytogenes* L7946 (arsenic-sensitive (-), cadmium-resistant (+)); *L. monocytogenes* L 7947 (arsenic-resistant (+), cadmium-sensitive (-)) and *L. innocua* L 2030c (tetracycline-resistant (+)).

VII.4.2 Differentiation of the Major *L. monocytogenes* Serotypes by Multiplex PCR

VII.4.2.1. DNA extraction

DNA extraction was performed according to Doumith *et al.* (2004): *L. monocytogenes* isolates were grown overnight on TSAYE at 37 ºC; 5-10 colonies were further ressuspended in 50 µL of a solution of 0.25% (w/v) sodium dodecyl sulphate (SDS)-0.05 N NaOH. The solution was heated at 99 ºC for 15 min. in a water-bath. One hundred µL of sterile Ultra pure (UP) water were added to the mixture, which was mixed by pipeting. Two µL of this mixture were used for PCR reaction.

VII.4.2.2 *Multiplex PCR*

Amplification reactions were performed in a final volume of 25 µl containing 0.5 U (µL) *Taq* DNA polymerase (Fermentas, Ontario, Canada), 0.2 mM deoxynucleoside triphosphates (ABgene, Epsom, United Kingdom) and 1 X PCR Buffer (Taq buffer +KCl -$MgCl_2$, Fermentas). The five primer sets (MWG Biotech, Convent Garden, London) were added at the following final concentrations: 1 µM for *lmo0737*, ORF2819 and ORF 2110; 1.5 µM for *lmo1118* and 0.2 µM for *prs*. PCR was performed with an initial denaturation step at 94 ºC for 3 min.; 35 cycles of 94 ºC for 0.40 min., 53 ºC for 1.15 min., and 72 ºC for 1.15 min.; and one final cycle of 72 ºC for 7 min. in a thermocycler (My-cycler, Bio-Rad Laboratories). 5 µL of the reaction mixture was mixed with 3 µl of loading buffer, 15 µl sterile UP water and separated on a 2% agarose gel at 80 mV in a TBE buffer (90 mM Trizma base, 90 mM boric acid, 2 mM EDTA, pH 8.3). The PCR products were visualized by ethidium bromide staining.

L. monocytogenes reference strains, from Collection de l´ Institute Pasteur, **R16** (serotype **1/2a), R13** (serotype **1/2b)**, **R11** (serotype **1/2c)** and **R1** (serotype **4b)**, were used as controls (Fig. 18)

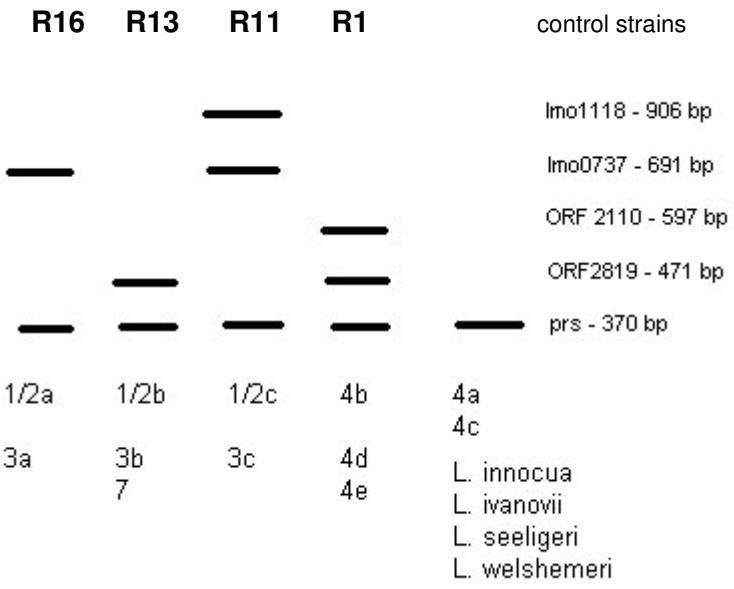

Fig. 18: Scheme of the different amplification products expected for each pair of primers.

Table 10: Nucleotide sequences of primer sets used in this study

Gene target	Primer sequence(5´-3´)[a]	Product size (bp)	Serotype specificity[b]	Protein encoded by the target gene
*lmo*0737	For:AGGGCTTCAAGGACTTACCC Rev: ACATTTCTGCTTGCCATTC	691	*L. monocytogenes* serotypes 1/2a, 1/2c, 3a and 3c	Unknown, no similarity
*lmo*1118	For:AGGGGTCTTAAATCCTGGAA Rev:CGGCTTGTTCGGCATACTTA	906	*L. monocytogenes* serotypes 1/2c and 3c	Unknown, no similarity
ORF2819	For:AGCAAAATGCCAAAACTCGT Rev:CATCACTAAAGCCTCCCATTG	471	*L. monocytogenes* serotypes 1/2b, 3b,4b,4d and 4e	Putative transcriptional regulator
ORF2110	For:AGTGGACAATTGATTGGTGAA Rev:CATCCATCCCTTACTTTGGAC	597	*L. monocytogenes* serotypes 4b,4d and 4e	Putative secreted protein
prs	For:GCTGAAGAGATTGCGAAAGAA G Rev:CAAAGAAACCTTGGATTTGCG G	370	All *Listeria* species	Putative phosphoribosyl pyrophosphate syntetase

[a] For, forward; Rev, reverse.;[b] For the specificity of *lmo 1118* gene fragment amplification within *L. monocytogenes* strains of serotype 1/2c or 3c, we note the exception of the serotype 1/2a EGDe strain n which the gene was first identified.

VII.4.3 Antibiotic susceptibility tests

L. monocytogenes isolates were subjected to antimicrobial sensitivity tests using the agar dilution method described in the guidelines of the National Committee for Clinical Laboratory Studies (NCCLS, 2004). Each isolate was tested for each antimicrobial agent. The antibiotics investigated were penicillin G, rifampicin, vancomycin, gentamicin, tetracycline, nitrofurantoin, ciprofloxacin and erythromycin. The stock solutions and the several dilutions of each antibiotic were prepared using recommended solvents and diluents (NCCLS, 2004). Two-fold dilutions ranging from 0.125 to 512 µg/mL for each antimicrobial agent were prepared, except for vancomycin (0.125-600 µg/mL). Two mL of each dilution were incorporated into a Petri dish containing 18 mL of Muller-Hinton agar (2-5% of lysed horse blood) at ca. 48 ºC. For penicillin G, Muller-Hinton Cation-Adjusted (BioMérieux SA) was used. For the preparation of the cellular suspension an overnight culture on TSAYE plates was resuspended in sterile Ringer's (LabM, Bury, U.K.) solution in order to obtain turbidity equivalent to 0.5 McFarland standard. The agar plates were inoculated with 1 µL of the bacterial suspension. Plates were incubated at 37 ºC for 24 hours. Plates without antibiotics were used as negative controls and *S. aureus* ATCC 25923 and/or *Enterococcus faecalis* ATCC 29212 were used as positive controls. For each antibiotic, susceptibility determination was performed at least in duplicate experiments.

VIII. RESULTS

In this chapter, the results of the different laboratorial techniques performed will be presented.

VIII.1 Positive samples (*L. monocytogenes*)

Results obtained for the detection and enumeration of *L.monocytogenes* using different techniques are presented in table 11. *L. monocytogenes* was detected in two of the 166 raw milk samples analysed, both collected in the same dairy farm, in Barcelos: one from the bulk tank milk, and the other from a clinical mastitis.

Table 11: Positive samples for the presence of *L. monocytogenes*, and enumeration results.

Region	Dairy Farm	Sample type	Direct Enumeration (cfu/mL)		MPN/mL	VIDAS
			Palcam	ALOA		
Barcelos	17	Bulk tank	< 1.5 x 10^1	< 1.0 x 10^1	< 20	+
		Clinical Mastitis	2.4 x 10^1	5.8 x 10^1	20	+

The silage, *unifeed,* cattle manure and water samples, as well as all the environmental samples collected in the "positive dairy farm" were negative for the detection and enumeration of *L. monocytogenes.*

L. innocua was identified in 42 (25.3%) of the raw milk samples: 16 bulk tank samples, 6 sub-clinical mastitis samples, 4 clinical mastitis samples and 16 healthy cows. *L. innocua* was also present in the two *L. monocytogenes* positive samples.

VIII.1.1 Characterization of *L. monocytogenes* isolates

From the two positive samples, 22 colonies were isolated and identified as *L. monocytogenes*:

- 2 from the bulk tank sample (1 from VIDAS positive tube after culture in ALOA and the other from PALCAM plates used in the enumeration procedure);

- 20 from the clinical mastitis sample (1 from MPN tubes isolated in PALCAM, 9 from MPN tubes isolated in ALOA, 9 from direct enumeration in ALOA and 1 from VIDAS positive tube after culture in ALOA).

Subtyping methods (resistance to arsenic, cadmium and tetracycline, antibiotic susceptibility tests and multiplex PCR) were used in order to investigate possible relationship between isolates (McLauchlin *et al.*, 1997). This is also particularly important since samples from foods or the environment can be contaminated simultaneously by multiple strains of *L. monocytogenes* (Vaz-Velho *et al*., 2001)

VIII.2 Resistance to Arsenic, Cadmium and Tetracycline

All *L. monocytogenes* isolates were sensitive to Arsenic, Cadmium and Tetracycline.

VIII.3 Antibiotic Susceptibility Tests

Table 12 summarizes the Minimum inhibitory concentrations (MIC), calculated for each *L. monocytogenes* isolate. With the exception of gentamycin, MICs for all the other antibiotics tested were very similar for all the isolates evaluated.

Table12: Distribution of MIC values for *L. monocytogenes* isolates by antimicrobial agent concentration.

Agent	Antimicrobial agent concentration µg/mL												
	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	›512
Erythromycin	0	5	17	0	0	0	0	0	0	0	0	0	0
Rifampicin	0	0	0	18	4	0	0	0	0	0	0	0	0
Gentamicin	17	0	0	5	0	0	0	0	0	0	0	0	0
Nitrofuratoin	0	0	0	0	0	0	0	0	0	0	0	0	22
Penicillin G	0	0	0	12	10	0	0	0	0	0	0	0	0
Vancomycin	0	0	0	1	20	1	0	0	0	0	0	0	0
Tetracycline	0	0	0	0	0	22	0	0	0	0	0	0	0
Ciprofloxacin	0	0	0	0	22	0	0	0	0	0	0	0	0

VIII.4 Multiplex PCR

According to the results obtained by the Multiplex PCR (fig.19), all the isolated strains were included in serogroup 4, which includes serotypes: 4b, 4d or 4e.

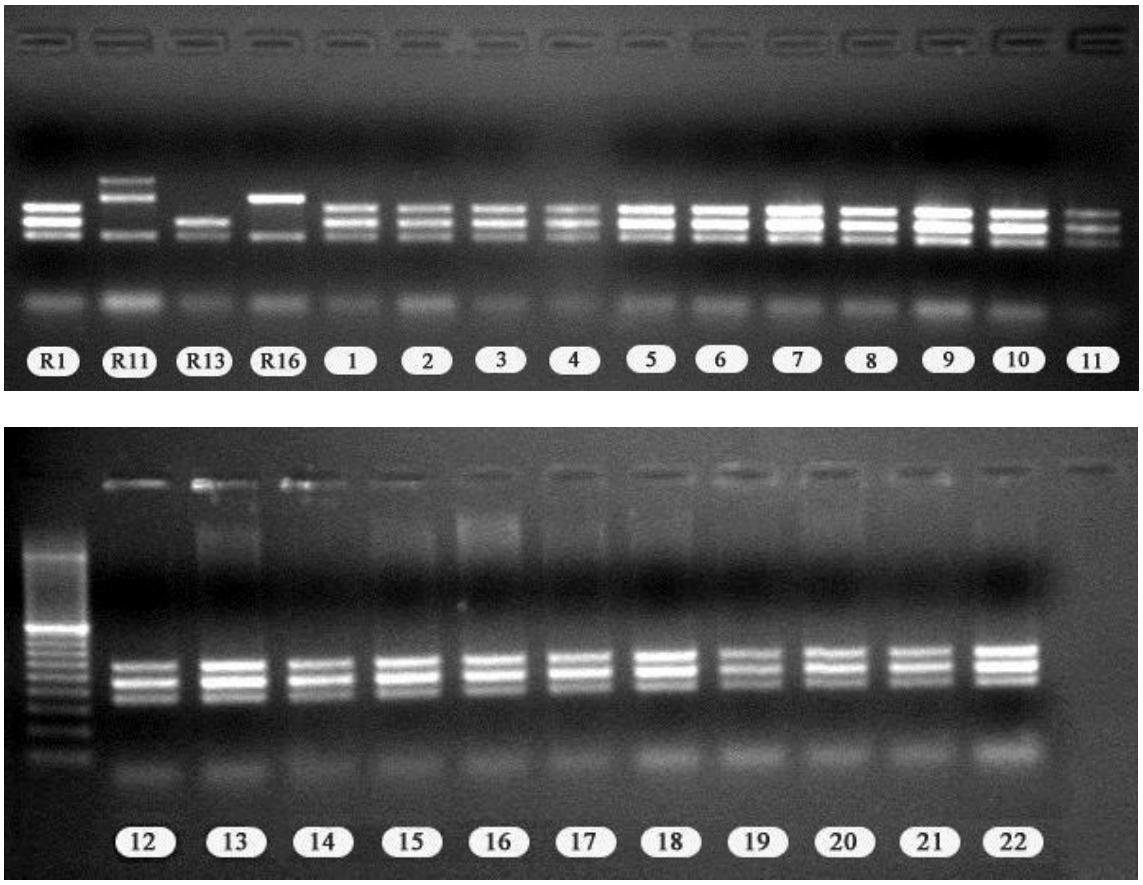

Fig. 19 **:** Agarose gel electrophoresis of DNA fragments generated by multiplex PCR with the serotyping reference strains of the species *L. monocytogenes* and the isolates of this study.
Legend: Lane **R1**, reference strain of serotype **4b**; Lane **R11**, reference strain of serotype **1/2c**; Lane **R13**, reference starin of serotype **1/2b**; Lane **R16**, reference strain of serotype **1/2a.** Lanes 1 and 2, isolates from the bulk tank sample; Lanes 3-22, isolates from the clinical mastitis sample.

IX. DISCUSSION

The results of this study suggest that *L. monocytogenes* has a low occurrence in cow's raw milk collected in the Portuguese Northern coast Region. Therefore, it does not seem that it should be considered as a major etiologial mastitis agent, but should although be considered as a biologial hazard in a dairy HACCP plan.

Having in mind that *L. monocytogenes* was isolated from only one dairy farm and that the results for the subtyping methods tested were almost the same for all *L. monocytogenes* isolates (small differences in MIC values, with the exception of gentamicin), this might suggest that the type identified is particularly adapted to the environment of that farm, and that the origin of the bulk tank contamination could be an infected cow, excreting *L. monocytogenes* through the udder.

There are however several aspects that should be considered and discussed:

IX.1 Sampling

According to Levy and Lemeshow (1980), sample size calculation could have been done using the following formula:

$$n = \frac{(Z^2)(N)(P_y)(1-P_y)}{(Z^2)(P_y)(1-P_y)+(N-1)(\varepsilon^2)(P_y)}$$

where n was the sample size, N was the size of the target population, P_y was the expected prevalence of *L. monocytogenes* based on previous studies, Z is the Z-value for the selected α level (for α=0.95, Z=1.96), and ε was an estimated value by which the sample estimate should not depart from the true population prevalence (0.1). The number of dairy farms in Portugal is decreasing over the last decade, but "Entre Douro e Minho" is still the leading region in terms of milk production. In 2004/2005 there were 4,460 dairy farms in this region (Hipólito *et al.*, 2006). Based on previous studies, expected prevalence of *L. monocytogenes* could be expected to be around 5%. So, based on the above formula, and considering N as a target population of 4,000 dairys, around

335 farms (bulk tanks) would be necessary to estimate the prevalence of *L. monocytogenes,* in the "Entre Douro e Minho" region.

Samples tested in this study were mainly collected during routine visits to evaluate milk quality. This might have influenced the results, as most of the visited dairy farms already have a milk quality information background, and, as previously refered, milking practices have a relation with *L. monocytognes* isolation (Hassan *et al.,* 2001). Although, the exact importance of this fact is difficult to be determined.

Besides this, the field part of the study took only 6 months, a limited time interval to evaluate a seasonal difference in the *L. monocytogenes* isolation.

IX.1.1 Mastitis sampling

In order to do the three techniques used to detect and enumerate *L. monocytogenes* in this study, we needed samples of about 80 mL. Usually, milk samples taken by the dairy producers have a lower volume (about 15 mL). This difference did not allow the use in this study of the milk samples that we get in the clinic. That would had been valuable to better evaluate *L. monocytogenes* as a mastitis aetiological agent. Results on blood agar could be compared with results obtained by conventional methods for *L. monocytogenes* detection, and it would had been possible to check if besides *L. monocytogenes* there was, at the same time, another bacterial agent.

IX.2 Detection and Enumeration procedures

According with the ISO procedures, it takes, at least 6 days (Waak *et al*., 1999), since a sample starts to be processed, until we get the species confirmation. According to Scotter *et al*. (2001) the method described in ISO 11290-2 has an overall sensitivity of 85.6% and a specificity of 97.4%, but in order to be able to analyse a higher number of samples maybe it would be helpful to use other (faster) techniques, or determine the enumeration only on the VIDAS-positive samples.

PCR-based methods are believed to have a great potential to fulfil the requirements for fast, specific and sensitive detection of *L. monocytogenes* in food. However, this potential may come to practical use only if appropriate sample preparation is used prior to PCR. The sample preparation should produce a sufficient amount of amplifiable DNA originating in live but not in dead *L. monocytogenes* cells. For this

reason, enrichment by culture seems a good choice, contrary to physical or immunomagnetic methods which do not distinguish between live and dead bacterial cells. Besides this, the detection limit of PCR is $\geq 10^4$ cfu/mL of *L. monocytogenes* (Kaclíková *et al*., 2003).

Recent PCR techniques have been described by:

- Rossmanith *et al*. (2006): Detection of *L. monocytogenes* in food using a combined enrichment/real-time PCR method targeting the *prfA* gene.

- Amagliani *et al*. (2004): Direct detection of *L. monocytogenes* from milk by magnetic based DNA isolation and PCR. The detection limit of this method is sufficient for direct detection of *L. monocytogenes* DNA in milk avoiding the enrichment culturing step, reducing the time necessary to obtain results from samples to 7 hours rather than the 5-day minimum required for the standard procedure.

Alternative techniques have been proposed by:

- Chemburu *et al.* (2005): Detection of pathogenic bacteria in food samples using highly-dispersed carbon particles, that allows detection and quantification of *L. monocytogenes*, with the detection limit of 10 cells/mL, and an overall assay time of 30 min.

- Peng & Shelef (2000): Rapid detection of low levels of *Listeria* in foods and next-day confirmation of *L. monocytogenes.* It consists of a 6-hours pre-enrichment step followed by overnight incubation in selective broth at 35 ºC. Changes in light transmittance in the selective broth are registered continuously by an optical sensor, and recorded in the computer. Aesculin hydrolysis by listeriae results in black coloration of the media that causes a sharp drop in light transmittance, whereas negative samples remain colorless. Confirmation of *L. monocytogenes* is carried out only on esculin-positive samples and is completed within 6 hours.

IX.3 Number of positive samples (for *L. monocytogenes*)

Only 1.2% of the samples analysed in this study were positive for the presence of *L. monocytogenes,* which is a percentage similar to that refered in international reports (Jensen *et al*., 1996; Kozak *et al.,* 1996; Gaya *et al*., 1996; Vitas *et al.* 2004). Lafarge *et al*. (2004) refers that the presence of metabolic substances produced by certain microorganims (lactic acid, hydrogen ions, bacteriocins, fatty acids), inhibit the growth of *L. monocytogenes.* This may be one of the possible explanations for such low occurrence, as milk contains a natural flora, and in mastitis samples there is (usually) a bacterial agent. The presence of other microorganisms was not tested, although, in this study. The presence of *L. innnocua* may be another factor that might turn difficult the detection of *L. monocytogenes.* This will be discussed in the next section.

IX.4 Association *L. monocytogenes* and *L . innocua*

It has been suggested that *L. monocytogenes* and *L. innocua* share the same ecological *niche* and therefore *L. innocua* could be used as an indicator strain for the presence of *L. monocytogenes* (Kozak *et al*., 1996; Dhanashree *et al*., 2003).

Scotter *et al*. (2001) found a significant number of false-negative results for the detection of *L. monocytogenes* when large numbers of *L. innocua* were present in the test materials (fresh cheese, minced beef and dried egg powder). *L. innocua* tended to dominate *L. monocytogenes* during the selective enrichment stages and thus masked small numbers of *L. monocytogenes* on the isolation media (Scotter *et al.,* 2001). In the present study, *L. innocua* was found in 25.3% of the samples. This represents a high percentage and could be an explanation, at least partially, for the low number of *L. monocytogenes* positive samples. However, the real significance of the presence of *L. innocua* in raw milk cannot be evaluated from the results obtained in this study.

IX.5 Refrigeration

Because of the large number of days necessary to confirm *L. monocytogenes*, and to the limited amount of time avaiable for this study, milk samples were stored up to 7-10 days prior to testing. The severity of damage done to bacterial cells by the length of refrigeration is difficult to ascertain, but it should be emphasided that milk was

documented as a good cryoprotectant (El-Kest & Marth, 1991). In fact, it probably protects better than glycerol, tryptose broth or phosphate buffer (El-Kest & Marth, 1991). In accordance with these ideas, Papageorgiou *et al*. (1997) concluded that the survival rate of *L. monocytogenes* can be as high as 95% after 7 months of storage, and Murdough *et al.* (1996) concluded that freezing of quarter milk samples for 6 weeks did not affect viability of any of the pathogens studied (*Strep*. spp, *Staph*. spp, *Corynebacterium bovis* and *E. coli*). On the other hand, Lafarge *et al*. (2004) examined 10 raw milk samples before and after 24 hours of storage at 4 ºC, and concluded that considerable evolution of bacterial populations occurred during conservation at 4 ºC, specifically, in *L. monocytogenes.* This "cold enrichment" does not seem to have occurred in this study, having in mind the low number of *L. monocytogenes* positive samples.

IX.6 Freezing/ Defrosting

Having in mind the intracellular facultative nature of *L. monocytogenes*, freezing/defrosting before detection/enumeration were evaluated. This technique had demonstrated to be useful for *S. aureus* diagnosis (Sol *et al*., 2002), when no growth was detected following the conventional technique. Interestingly, the bulk tank that was positive for the presence of *L. monocytogenes* before freezing was negative in the sample analysed after freezing and defrosting. It should, however, be emphasised that these samples were from the same bulk tank, were taken in the same day, but they cannot be considered the same sample considering the volume of the tank and the probability of finding *Listeria*. In the other 13 samples "double-analysed", *L. monocytogenes* was not isolated neither before nor after freezing, indicating that perhaps freezing/defrosting may not be an useful technique for the detection of false negative *L. monocytogenes* results.

IX.7 Origin of animal contamination

Silage as been refered as an usuall source of *L. monocytogenes* animal contamination (Amstutz, 1980). The results for detection and enumeration of *L. monocytogenes* in silage sample analysed in this study were negative, but this does not mean that the all silo was negative for the presence of *L. monocytogenes*. As in *unifeed*

and water samples, *L. monocytogenes* was not also detected, the origin of animal contamination can not be refered for this particular case.

IX. 8 Antibiotic susceptibility

Specific *Listeria* breakpoints are only defined for ampicillin (MIC ≥ 2 µg/mL) and penicillin G (MIC ≥ 2 µg/mL) (Srinivasan *et al*., 2005). For the other antibiotics tested, breakpoints used were those recommended by NCCLS (2004) for other Gram-positive microorganisms:

- Erythromycin: MIC ≥ 1 µg/mL
- Gentamicin: MIC ≥ 8 µg/mL
- Rifampicin: MIC ≥ 4 µg/mL
- Tetracycline: MIC ≥ 16 µg/mL
- Vancomycin: MIC ≥ 32 µg/mL
- Ciprofloxacin: MIC ≥ 4 µg/mL
- Nitrofurantoin: MIC ≥ 128 µg/mL

According with these values, all the *L. monocytogenes* isolates of this study can be classified as susceptible to all the antibiotics tested, except for Nitrofurantoin, although some of the MICs are close to the breakpoint value. This is of particular importance because although *L. monocytogenes* was noted to be relatively susceptible to a wide range of antimicrobials as few as 15 years ago, a number of more recent reports suggest that the rate of antimicrobial resistance in *L. monocytogenes* is increasing (Teuber, 1999; Srinivasan *et al.*, 2005).

IX.9 Serotype

As refered (Lundén *et al*., 2004), there is a discrepancy between clinical and food isolates, as the most common serotype in European listeriosis outbreaks has been 4b, and serogroup 1/2 is the leading serogroup in foods. According to the results obtained by the Multiplex PCR (fig.19), all the isolated strains in this study can only be included in serogroup 4 (which includes, beside others, serotypes 4b, 4d and 4e), so additional studies are needed to determine the specific serotype isolated. Anyway, the serotype

isolated in this study does not belong to the leading serogroup in foods. Interestingly, in a recent study by Chambel *et al.* (2007), that checked the occurrence and persistence of *Listeria* spp. in the environment of ewe and cow's milk cheese dairies in Portugal, 52% of genomic types were also serotype 4b.

X. CONCLUSION

The challenges to provide a safe and nutritious food supply are complex because all aspects of food/milk production need to be considered. Given the considerable national/international demand for food safety and the formidable challenges of producing and maintaining a safe food supply, food safety research and educational programs has taken on a new urgency. As the system of food production and distribution changes, the food safety system needs to change with it. A strong science-based approach that addresses all the complex issues involved in continuing to improve food safety and public health is necessary to prevent foodborne illnesses. Research and educational efforts identifying potential on-farm risks will better enable dairy producers to reduce/prevent foodborne pathogen contamination of dairy products leaving the farm. Research must not only be conducted to solve complex food safety problems, but results of that research must be communicated effectively to dairy farmers and consumers.

Additional studies are needed to verify our results, to identify risk factors for the presence of *L. monocytogenes* in raw milk, and to assess the public health impact. However, conclusions from this study support the hypothesis that Foodborne Pathogens, Mastitis, Milk Quality and Dairy Food Safety are indeed all interrelated. A safe, abundant and nutritious milk and meat supply should be the goal of every dairy producer in the world, and as a veterinarian, I hope that this thesis could be a helpful tool to achieve this purpose.

XI. FURTHER WORK

After finishing this study, and having in mind its results/conclusions, there are several aspects that could be further developed. Some ideas and suggestions for future work:

- Sample a larger number of dairy farms/cows, over a larger period of time (to evaluate a possible seasonal variation), and involving a broader geographical area; In that study, one of the recently developed and refered methods to detect *L. monocytogenes* could be used, not only to be able to analyse a bigger number of samples, but also to have faster results, something essential for the clinical samples;
- Develop a project, with the aim to determine how significant the misdiagnosis of *Listeria* spp. is at the moment in a clinical laboratory, having in mind the possible confusion with *Corinebacterium* spp. and yeasts.
- Try to find the origin of the animal contamination in this study, anylising, for example, rodents (if present) faeces and more silo samples.
- Further charachterisation of the *L. monocytogenes* isolates of this study should be done, using, for example Random Amplified Polymorphic DNA (RAPD) PCR or PFGE.
- Evaluate antibiotic susceptibility of the *L. monocytogenes* isolates of this study to ampicillin (as it is one of the first therapeutical choices in Humans) and other β-lactamic antibiotics.
- Develop a study, using only milk samples previously cultured in routine bacteriological media, with negative growth results, and try to find new solutions for this diagnosis problem.

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