UNIVERSIDADE TÉCNICA DE LISBOA

Instituto Superior de Agronomia

Efeito de bioconservantes no crescimento e sobrevivência de *Listeria monocytogenes* em queijo de ovelha

Doutoramento em Engenharia Agro-Industrial

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Tese apresentada neste instituto para obtenção do grau de doutor em Engenharia Agro-Industrial

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Aos meus pais

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BEM-HAJAM

RESUMO

Estirpes de *L. monocytogenes*, *L. innocua* e *L. seeligeri,* isoladas em duas regiões produtoras de queijo de ovelha (Castelo Branco e Tolosa) foram caracterizadas. Com base na tipagem molecular (AFLP, PFGE e serotipagem molecular) foi possível estabelecer a associação entre um caso de listeriose ovina assintomática e a contaminação de queijo feito com leite cru e do ambiente, tendo-se encontrado o mesmo tipo molecular (AFLP IV-1, PFGE 11 e serogrupo molecular 4b). A caracterização molecular de 185 estirpes permitiu estudar as vias de contaminação e a persistência de *L. monocytogenes* dentro das queijarias. Nos testes de sensibilidade à nisina, foi determinada a Concentração Mínima Inibitória (CMI) de 50 IU de nisina / ml para a maioria das 219 estirpes analisadas e uma frequência média de resistentes de $1{:}10^4$. Foi avaliada a virulência (*Plaque-forming-assay* com células animais HT-29) de 7 estirpes de *L. monocytogenes*. Para controlar a presença de microrganismos patogénicos ou de alteração (*Listeria monocytogenes, Pseudomonas aeruginosa, Yarrowia lipolytica, Penicillium commune* e *P. chrysogenum*), naturalmente presentes na casca do queijo, foram desenvolvidos fluidos filmogénicos à base de isolado proteico de soro de leite de pH 3, tendo estes sido caracterizados quanto à sua acção antimicrobiana, propriedades viscoelásticas e de viscosidade e propriedades mecânicas e de permeabilidade ao vapor de água.

Palavras-chave: *Listeria monocytogenes*, revestimentos antimicrobianos, nisina, queijo, listeriose ovina, tipagem molecular

Effect of biopreservatives on growth and survival of *Listeria monocytogenes* in ewe´s cheese

ABSTRACT

Strains of *L. monocytogenes*, *L. innocua* and *L. seeligeri,* isolated from two regions producing ewe´s cheese (Castelo Branco and Tolosa) were characterized. Using molecular typage (AFLP, PFGE and molecular serotyping) it was possible to do the association between a case of asymptomatic ovine listeriosis and the contamination of cheese made with raw milk and the environment, as the same molecular type was revealed (AFLP IV-1, PFGE 11 and molecular serogroup 4b). The molecular characterization of 185 isolates permitted to study the routes of contamination and the persistence of *L. monocytogenes* within cheese processing plants. Applying tests of susceptibility to nisin, the Minimum Inhibitory Concentration (MIC) of 50 IU of nisin / ml for the majority of the 219 strains analyzed and a mean frequency of resistant of $1{:}10^4$ was calculated. The virulence of 7 *L. monocytogenes* strains was evaluated by a plaque-forming-assay, with HT-29 monolayer animal cells. To control the presence of pathogenic and spoilage microorganisms (*Listeria monocytogenes, Pseudomonas aeruginosa, Yarrowia lipolytica, Penicillium commune* and *Penicillium chrysogenum*), naturally present on the cheese rind, were developed filmogenic fluids based on whey protein isolate, with pH 3, having been characterized for their inhibitory effect, viscoelastic and viscosity properties, as well as for mechanical properties and water vapour permeability.

Key-words: *Listeria monocytogenes*, antimicrobial coatings, nisin, cheese, ovine listeriosis, molecular typage

ABREVIATURAS

AFLP – *Amplified Fragment Length Polymorphism*

ATCC – *American Type Collection Culture*

CFU – *Colony Forming Units*

CLIP – *Collection des Listeria de l´Institut Pasteur*

DOP – Denominação de Origem Protegida

%E - *Percentage of Elongation*

EDTA – *Ethylenediamine-Tetraacetic Acid*

FAO – *Food and Agriculture Organization of the United Nations*

For. ou F.- *Forward*

G - *Glycerol*

G´- *Storage Modulus*

G´´- *Loss Modulus*

GRAS – *Generally Recognized as Safe*

GYPA - *Glucose Yeast Peptone Agar*

HPA – *Health Protection Agency*

IP – *Isoelectric Point*

IU – *International Unit*

LD_{50} – *50% Lethal Dose*

MA – *Malic Acid*

MIC - *Minimum Inhibitory Concentration*

MM - *Molar Mass*

N - *Nisin*

NA - *Natamycin*

NCTC – *National Collection of Type Cultures*

PCR – *Polymerase Chain Reaction*

PFA – *Plaque Forming Assay*

PFGE –*Pulsed Field Gel Electrophoresis*

PM – Peso Molecular

PS - *Puncture Stress*

PT – *Pulsotype*

Rev. ou R.- *Reverse*

RH - *Relative Humidity*

RT-PCR – *Real Time-Polymerase Chain Reaction*

S - *Sorbitol*

SAOS - *Small Amplitude Oscillatory Stress*

SCC – *Somatic Cell Count*

Ser. - *Serovar*

TS - *Tensile Stress*

TSB – *Trypticase Soy Broth*

TSYEGA - *Tryptone Soy Yeast Extract Glucose Agar*

TSYEGB - *Tryptone Soy Yeast Extract Glucose Broth*

UPGMA – *Unweighted Pair Group Mean Arithmetic*

WHO – *World Health Organization*

WPC - *Whey Protein Concentrate*

WPI – *Whey Protein Isolate*

WVP - *Water Vapour Permeability*

ÍNDICE

AGRADECIMENTOS i
RESUMO iv
ABSTRACT v
ABREVIATURAS vi

CAPÍTULO I

Introdução geral

1. *Listeria monocytogenes* 2
    1.1. Características gerais 2
    1.2. Listeriose 3
    1.3. *Listeria monocytogenes* em produtos lácteos 6
    1.4. Virulência 8
    1.5. Tipagem molecular 10
        1.5.1. Métodos de tipagem 10
        1.5.2. Extracção de DNA 10
        1.5.3. *Taq-Man Real Time PCR* 11
        1.5.4. *Amplified fragment length polymorphism* 13
        1.5.5. Serotipagem molecular 15
        1.5.6. *Pulsed field gel electrophoresis* 18
    1.6. Sensibilidade de *Listeria* spp. a nisina 19
2. Revestimentos edíveis com propriedades antimicrobianas 22
    2.1. Características gerais dos revestimentos 22
    2.2. Revestimentos à base de isolado proteico de soro de leite 23
    2.3. Revestimentos com propriedades antimicrobianas 27
3. Enquadramento geral e objectivos 29
4. Referências bibliográficas 33

CAPÍTULO II

Association between a case study of asymptomatic ovine listerial mastitis and the contamination of soft cheese and cheese processing environment with *Listeria monocytogenes* in Portugal

Abstract 49
1. Introduction 50
2. Material and methods 51

2.1. Cheese manufacturers 51
2.2. Microbiological analyses, examination, and sampling procedures 52
2.3. Examination of sheep, and sampling procedures 52
2.4. Confirmation and subtyping of *L. monocytogenes* isolates 53
3. Results 55
4. Discussion 59
Aknowledgements 61
References 61

CAPÍTULO III

Routes of contamination by *Listeria monocytogenes* in ewe's milk cheese processing plants in Portugal

Abstract 67
1. Introduction 68
2. Material and methods 69
2.1. Cheese manufacturers 69
2.2. Sampling procedures 70
2.3. Detection and subtyping of *Listeria* isolates 70
2.4. Virulence evaluation of *Listeria monocytogenes* isolates 72
3. Results 72
3.1. Occurrence of *Listeria* spp. and subtyping of *Listeria* isolates 72
3.2. Routes of transmission 75
3.3. Evaluation of virulence 78
4. Discussion 79
Aknowledgements 80
References 81

CAPÍTULO IV

High nisin susceptibility of *Listeria* spp. wild-type strains isolated from dairies with traditional cheese preservation in Portugal

Abstract 85
1. Introduction 86
2. Materials and methods 87
2.1. Nisin and culture media 87
2.2. *Listeria* spp. strains 87
2.3. Minimum inhibitory concentration 88
2.4. Effect of nisin on *L. monocytogenes* CP6 in broth medium 88
3. Results 89

4. Discussion 92
Aknowledgements 95
References 95

CAPÍTULO V

Properties of whey protein based films containing organic acids and nisin to control *Listeria monocytogenes*

Abstract 100
1. Introduction 101
2. Materials and methods 102
 2.1. Preparation of film solutions and films 102
 2.2. Rheological measurements 103
 2.3. Mechanical properties of films 103
 2.4. Culture media and bacterial strains 104
 2.5. Antimicrobial agar diffusion assay 104
 2.6. Statistical analysis 104
3. Results and discussion 105
 3.1. Selection of type and concentration of organic acids 105
 3.2. Antimicrobial properties of films made with acids (3.0%) and nisin 108
 3.3. Mechanical and rheological properties of film solutions made with acids (3.0%) and nisin 110
Aknowledgements 114
References 114

CAPÍTULO VI

Control of pathogenic and spoilage microorganisms from cheese surface by whey protein films containing malic acid, nisin and natamycin

Abstract 118
1. Introduction 119
2. Materials and methods 120
 2.1. Materials used to produce films and specifications 120
 2.2. Microbial cultures and media 121
 2.3. Preparation of film solutions and films 123
 2.4. Antimicrobial diffusion-type assay 123
 2.5. Film thickness 124
 2.6. Water vapour permeability determination 124
 2.7. Mechanical properties of films 124
 2.8. Rheological measurements 125

2.9. Statistical analysis 125
3. Results and discussion 126
3.1. Antimicrobial activity 126
3.2. Viscosity and viscoelastic properties 130
3.3. Water vapour permeability 130
3.4. Mechanical properties 132
4. Conclusions 134
Aknowledgements 134
References 134

CAPÍTULO VII
Considerações finais e perspectivas futuras 139

ANEXO I
Table 1. Strains of *Listeria* isolated from soft cheese 144
ANEXO II
Publicações e comunicações 147

CAPÍTULO I

Introdução geral

1. *Listeria monocytogenes*

1.1. Características gerais

Foi em 1926 que o agente etiológico da listeriose foi descrito pela primeira vez de forma detalhada por Murray, Webb e Swann, quando estudavam sangue de coelhos infectados por um bacilo Gram-positivo, designado na altura por *Bacterium monocytogenes* (McLauchlin, 1987). A listeriose humana viria a ser mais tarde descrita por Nyfeldt em 1929, o qual isolou o agente de sangue de pacientes que sofriam de mononucleose, tendo este designado o agente por *Listerella monocytogenes hominis* (McLauchlin, 1987). Este nome foi usado até 1939, altura em que se descobriu que o nome do género *Listerella* já tinha sido atribuído a um grupo de *slime molds* em 1906. Em 1940, Pirie propõe a alteração do nome para *Listeria monocytogenes* (também em honra a Lister), o qual se manteve até aos nossos dias. Actualmente, para além da espécie *L. monocytogenes* o género *Listeria* compreende mais 5 espécies: *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri* e *L. grayi.*

De acordo com a análise filogenética levada a cabo recentemente por Schmid *et al*. (2005), a espécie *L. grayi* representa o ramo mais antigo do género *Listeria*, ao passo que as restantes espécies se separaram posteriormente em duas linhas evolutivas, uma contendo as espécies *L. monocytogenes* e *L. innocua* e outra englobando *L. seeligeri*, *L. welshimeri* e *L. ivanovii.*

As bactérias do género *Listeria* são bacilos pequenos e regulares, Gram-positivos, móveis por meio de 2 a 5 flagelos perítricos (a 20-25ºC), aeróbios ou anaeróbios facultativos, não esporulados e não formadores de cápsula. A mobilidade do tipo *tumbling* é uma característica que pode ser usada na sua identificação. A temperatura óptima para a sua multiplicação é 30-37ºC, mas conseguem crescer a temperaturas compreendidas entre 1-45ºC. Devido à sua capacidade para crescer a temperaturas inferiores a 5ºC é considerado um microrganismo psicrotrófico. O intervalo de pH que permite o crescimente é 5,6-9,6 e algumas estirpes toleram um valor máximo de 10% de NaCl (Seeliger e Jones, 1987).

1.2. Listeriose

A listeriose é uma zoonose, doença comum ao homem e animais, que se caracteriza essencialmente por casos esporádicos, seguindo-se os focos epidémicos e, por último, as infecções nosocomiais (Schelcher *et al.*, 1992).

Das diversas vias de infecção conhecidas (aérea, cutânea, conjuntiva, transplacentária, transmembranária, nosocomial, por contacto directo, digestiva), o consumo de alimentos contaminados é a principal, tanto para o homem como para os animais (McLauchlin *et al.*, 2004).

De acordo com Marth (1988) e Doganay (2003), os grupos de risco normalmente associados a esta doença são mulheres grávidas, fetos e recém-nascidos, pacientes com alguma patologia que comprometa as defesas imunitárias, pessoas idosas e ainda trabalhadores rurais, técnicos e médicos veterinários que contactam com animais infectados. Destes vários grupos destaca-se o dos idosos com mais de 65 anos de idade, uma vez que 55,6% dos casos de listeriose humana reportados na Europa estão a ele associados (EFSA, 2007a).

A maioria das pessoas infectadas com *L. monocytogenes* tem o seu sistema imunitário de mediação celular debilitado (Lorber, 1990). No entanto, 30 a 50% dos casos de listeriose humana ocorrem em pessoas sem qualquer imunodepressão conhecida.

Listeria é uma bactéria intracelular facultativa. Uma vez que consegue sobreviver e multiplicar-se no interior de diversos tipos de células eucariotas, consegue escapar à acção do sistema imunitário. Na ausência do controlo deste, a bactéria pode então facilmente disseminar-se e alcançar, por via sanguínea, a placenta ou o sistema nervoso central, partindo de focos de multiplicação intracelular.

A dose mínima de infecção de *L. monocytogenes* para o homem ainda não foi determinada, devendo no entanto ser elevada para os indivíduos saudáveis. Newton *et al.* (2005) estimam que a dose letal para o homem seja da ordem de 10^8 células de *L. monocytogenes* por grama de alimento.

As manifestações clínicas da listeriose humana são muito diversificadas e diferem tendo em conta a via infecciosa, a dose e o grupo de risco. Genericamente, incluem meningite, encefalite, septicemia, endocardite, mononucleose, aborto,

pneumonia, abcessos localizados, conjuntivite e uretrite. Na população saudável, o consumo de alimentos contaminados causa gastrenterite com febre, vómitos, náuseas e diarreia (Doganay, 2003). Em poucos instantes esta forma de listeriose pode, no entanto, tornar-se invasiva.

Cerca de 1% a 9% da população humana é portadora de *L. monocytogenes*, sendo alguns destes indivíduos assintomáticos (Ryser e Marth, 1991).

O desenvolvimento ou não de uma infecção provocada por *L. monocytogenes* depende de: (1) susceptibilidade e estado imunitário do hospedeiro; (2) quantidade de inóculo; (3) virulência da estirpe; (4) matriz alimentar (Daugelat *et al.,* 1994; McLauchlin *et al.,* 2004). Relativamente a este último parâmetro, a composição do alimento (teor em gordura, em sal, em ácido e em água, por exemplo) vai modular a capacidade desta bactéria para sobreviver no interior do corpo e expressar a sua virulência (McLauchlin *et al.,* 2004).

Só a partir dos anos 80 foi evidenciada a acção de *Listeria monocytogenes* como agente patogénico de origem alimentar. Desde essa altura esta bactéria tem estado associada a vários surtos de listeriose por ingestão de alimentos contaminados, com uma taxa de mortalidade de 20-30%. Esta situação tem preocupado um número crescente de responsáveis de saúde pública em todo o mundo. De acordo com Mead *et al.* (1999), a listeriose foi considerada nos USA a segunda maior causa de morte de origem alimentar, logo a seguir à salmonelose. Ainda de acordo com a mesma fonte, os microrganismos pertencentes aos géneros *Listeria*, *Salmonella* e *Toxoplasma* são responsáveis, cada ano, por mais de 75% do total de mortes provocadas pelo consumo de alimentos contaminados nos USA, entre os 28 agentes microbianos considerados neste estudo (19 bactérias, 5 parasitas e 4 vírus).

Os membros do género *Listeria* estão largamente distribuídos no ambiente, tendo sido já isolados do solo, vegetação, águas de superfície, águas de esgoto, diversos tipos de alimentos e respectivos ambientes fabris. Também se podem igualmente encontrar no conteúdo intestinal de 4% a 30% de humanos e animais saudáveis (Rocourt, 1992). Facilmente se compreende assim que as deficientes condições higiénicas de produção e manipulação de alimentos tornem a sua contaminação um acontecimento de elevada probabilidade. Uma vez o alimento contaminado por bactérias do género *Listeria*, a sua sobrevivência e multiplicação

ficam dependentes da interacção de vários factores ambientais e nutricionais, incluindo a temperatura, pH, pressão de oxigénio e concentração de cloreto de sódio (Buchanan *et al*., 1989).

As características do género *Listeria* ajudam a compreender a origem alimentar da listeriose humana. Apesar de ser um microrganismo não esporulado, estas bactérias são extremamente resistentes às condições ambientais. É considerada uma bactéria ubíqua, consegue sobreviver aos processos de higienização nas indústrias alimentares e consegue sobreviver e mesmo multiplicar-se em alimentos com elevadas concentrações de sal e numa ampla gama de temperaturas, incluindo a temperatura de refrigeração (Lundén *et al*., 2003). Importa também referir que estas bactérias sobrevivem em alimentos com pH baixo, e que a adaptação a estes meios de baixo pH pode induzir protecção cruzada relativamente a vários factores adversos presentes no queijo, como é o caso de elevadas concentrações de NaCl (Faleiro *et al*., 2003).

Dos vários registos existentes de casos de listeriose provocados pelo consumo de alimentos contaminados com *Listeria monocytogenes,* salientam-se os seguintes, por ter ficado estabelecida a sua origem no consumo de queijo (Carrique-Mas *et a*l., 2003; Gaulin *et al*., 2003; McLauchlin *et al*., 2004; MacDonald *et al*. 2005; Makino *et al*., 2005; Mead *et al*., 2006; Gillespie *et al*., 2006; Bille *et al*., 2006; Vit *et al*., 2007, EFSA, 2007a): Califórnia / USA em 1985 (30 mortes / 142 casos), Waadt / Suiça entre 1983 e 1987 (34 mortes / 122 casos), França em 1995 (4 mortes / 17 casos), Reino Unido em 1999 (1 morte / 2 casos), USA em 2000 e 2001 (5 mortes / 12 casos), Suécia em 2001 (50 casos), Japão em 2001 (38 casos), Canadá em 2002 (17 casos), Suíça em 2005 (10 casos), República Checa em 2006 (78 casos), Alemanha em 2006 (6 casos).

Apesar da exposição à bactéria ser comum, a listeriose é uma doença rara. De acordo com de Valk *et al.* (2003), a incidência de listeriose humana na Europa varia entre 1 e 7 casos por milhão de habitantes e por ano. Em Portugal, a listeriose não é uma doença de declaração obrigatória, como acontece em países como USA, França, Alemanha, Reino Unido, Canadá e Suiça, e os dados existentes são escassos. Só recentemente foi divulgada informação que sugere que a listeriose humana em Portugal apresenta níveis semelhantes aos publicados para os restantes países (Almeida *et al*., 2006).

Apesar de Portugal integrar desde 1990 o Programa de Vigilância e Controlo das Toxinfecções Alimentares Colectivas da Região Europeia da OMS, para o qual tem que contribuir com dados nacionais, este não foi ainda implementado com resultados práticos. Este programa está integrado no Plano Nacional de Controlo da Higiene Alimentar, da responsabilidade da Direcção Geral de Saúde, e foi instituído através da Circular Normativa 14/DT de 09/10/2001 (Direcção-Geral de Saúde, 2004).

As toxinfecções alimentares colectivas são consideradas um problema de saúde pública e a sua vigilância epidemiológica é fundamental para o sucesso de uma política integrada de segurança alimentar, permitindo a intervenção baseada na evidência. Torna-se assim urgente pôr em prática o programa de vigilância que já existe.

1.3. *Listeria monocytogenes* em produtos lácteos

Os produtos lácteos são particularmente susceptíveis de sofrerem contaminações por este microrganismo, face à frequência com que *L. monocytogenes* ocorre nos estábulos, equipamentos de ordenha e ambiente das fábricas de lacticínios (Knight *et al.*, 1988). Esta bactéria tem sido isolada no leite cru (Lovett *et al.*, 1987; Harvey e Gilmour, 1992; Manzano *et al.*, 1997; Pereira e Pintado, 2000), no leite pasteurizado (Garayazabal *et al.*, 1986), no leite em pó (Doyle *et al.*, 1985), nos queijos (McLauchlin *et al.*, 1990; Carminati *et al.*, 2004; Pintado *et al.*, 2005), no iogurte (Shaack e Marth, 1988), em gelados e em manteiga (Schelcher *et al.*, 1992; Gillespie *et al.*, 2006). No entanto, do conjunto de produtos referidos, os queijos de pasta mole e semi-mole são os que têm oferecido uma maior percentagem de isolados de *Listeria* (Rocourt *et al.*, 2003).

A elevada prevalência de *Listeria monocytogenes* em produtos de origem láctea, assim como a ocorrência de vários casos de listeriose associados ao consumo de queijos de pasta mole, apontam para a importância destes alimentos como veículos de transmissão da doença (Zottola e Smith, 1991). De acordo com as propriedades definidas pelo *International Life Sciences Institute* (ILSI, 2005), o queijo encontra-se na categoria dos alimentos de alto risco, visto que: (1) tem

potencial para ser contaminado por *L. monocytogenes*; (2) suporta o crescimento de *L. monocytogenes* em elevados números; (3) é um alimento pronto-a-comer; (4) requer refrigeração; (5) é conservado durante longos períodos de tempo.

A presença de *L. monocytogenes* no queijo pode ser atribuída ao facto de estes serem muitas vezes fabricados com leite cru contaminado, a contaminação ambiental durante o processo de fabrico e maturação ou ainda a contaminação pós-processamento (Zottola e Smith, 1991). *L. monocytogenes* pode estabelecer-se no ambiente das queijarias, tornando-se uma fonte de contaminação durante a produção dos queijos. Este facto tem sido confirmado através do uso de métodos de tipagem molecular (Waak *et al.*, 2002; Kabuki *et al.*, 2004; Danielsson-Tham *et al.*, 2004).

Os ruminantes em lactação podem excretar por longos períodos de tempo *L. monocytogenes* no leite como consequência da presença de mamites. As mamites clínicas e subclínicas nas ovelhas são uma afecção de elevada prevalência que causa perdas importantes nos rebanhos, mantendo-se muitas vezes a infecção durante o período seco. A qualidade do leite é fortemente depreciada e a saúde pública posta em causa. Para além das perdas produtivas, produção de leite com propriedades tecnológicas alteradas e quebras de crescimento das crias ou mesmo a morte do animal, há ainda a salientar que um animal com mamite subclínica constitui um reservatório camuflado de microrganismos potencialmente patogénicos, dado que a sua detecção escapa a um normal exame visual. Acresce ainda o facto de alguns animais serem portadores assintomáticos e excretarem a bactéria nas fezes (Rea *et al.*, 1992; Schöder *et al.*, 2003).

Evitar a contaminação da matéria-prima e dos alimentos processados por *L. monocytogenes* deverá ser uma das principais estratégias de controlo a preconizar para se conseguir uma redução do número de casos de listeriose. No caso particular do queijo, tanto as explorações produtoras de leite como as queijarias precisam de trabalhar em conjunto, implementando Boas Práticas de Maneio e Boas Práticas de Produção (Tompkin, *et al.*, 1999; Tompkin, 2002), respectivamente. Torna-se igualmente necessária a implementação dos princípios do sistema de segurança alimentar "Análise de Perigos e Pontos Críticos de Controlo" (HACCP), um sistema abrangente que vai desde a produção da matéria-prima até ao consumidor final.

1.4. Virulência

Entre as 6 espécies de *Listeria*, apenas *L. monocytogenes* e *L. ivanovii* são consideradas virulentas (Roche *et al*., 2001). Todas as espécies não hemolíticas e a *L. seeligeri*, espécie ligeiramente hemolítica, são avirulentas nos testes de patogenicidade com camundongos (McLauchlin *et al*., 2004). A inoculação de *L. monocytogenes* por via subcutânea, intraperitonial, intravenosa ou oral causa a morte destes animais num período de 1 a 7 dias. Após inoculação por via intraperitonial ou intravenosa, a dose letal LD_{50} para os camundongos situa-se entre 10^2 e 10^7 bactérias.

A virulência de *L. monocytogenes* é uma propriedade condicionada pelo menos por 9 genes e pelos seus produtos (Tabela 1), os quais são necessários para a adesão / infecção, invasão, sobrevivência, mobilidade e propagação célula a célula (Vazquez-Boland *et al*., 2001). Os mesmos autores referem ainda a reduzida variabilidade nos factores de virulência existente entre as diferentes estirpes de *L. monocytogenes.* Para além disso, o conjunto dos genes de virulência é muito semelhante nas espécies *L. monocytogenes, L. ivanovii* e *L. seeligeri*. Sabe-se também que os genes envolvidos na invasão e movimento intracelular nas células dos mamíferos estão localizados em operões adjacentes ou em locais muito próximos do cromossoma bacteriano.

Tabela 1. Principais factores de virulência do género *Listeria*.

Actividade	Factores de virulência
Actin-based motility; propagação célula a célula	ActA
Adesão	Ami
Metabolismo celular	Hpt
Exoenzimas	Mpl; fosfolipases (PlcA, PlcB)
Invasão intracelular	Internalinas (InlA, InlB)
Regulação	PrfA
Proteínas de stress, que permitem a adaptação a ambientes adversos	ClpC, ClpE, ClpP (proteases)
Toxina que lisa a membrana do vacúolo	Listeriolisina O (LLO)
Não atribuída	SvpA; p60 (hidrolisa a mureina na fase final da divisão celular)

Fonte: Doyle (2001); Vazquez-Boland *et al.* (2001); Jaradat e Bhunia (2003)

Apesar dos casos de listeriose humana estarem associados à bactéria *L. monocytogenes*, sabe-se que nem todas as suas estirpes são patogénicas, havendo níveis heterogéneos de virulência (Gracieux *et al*., 2003). Estima-se que cerca de 10% a 20% de estirpes *L. monocytogenes* são não-patogénicas ou são fracamente patogénicas. Roche *et al.* (2001) encontraram 16% de estirpes avirulentas ou hipovirulentas entre as estirpes não clínicas de *L. monocytogenes* analisadas. As estirpes de baixa virulência são mais abundantes nos alimentos do que no ambiente (Roche *et al*., 2001).

A Listeriolisina O é um importante factor de virulência na patogenicidade de *L. monocytogenes* mas uma avaliação quantitativa deste factor não permite determinar, por si só, o potencial patogénico das estirpes. Os ensaios *in vitro* em que a avaliação da virulência das estirpes é baseada no uso de marcadores genotípicos ou fenotípicos da virulência ou no uso de linhas celulares intestinais (TCA, *tissue culture assays*) (van Langendonck *et al*., 1998; Roche *et al*., 2001) e os estudos *in vivo* com modelos animais (van Langendonck *et al*., 1998) são importantes para a confirmação das características de patogenicidade. Os ensaios *in vivo* levantam questões de ordem ética, são caros e morosos. Entre os TCA desenvolvidos com linhas celulares animais, destaca-se o teste de virulência implementado por Roche *et al.* (2001), em que se observa a formação de placas (PFA, *plaque-forming assay*) após inoculação da estirpe de *L. monocytogenes* sobre uma monocamada de células animais HT-29, linha celular proveniente de tecido de adenocarcinoma humano. Este teste fornece resultados reprodutíveis em 24 horas. Para além disso, considera a acção conjunta de todos os genes associados à virulência, indispensáveis para que determinada estirpe seja considerada patogénica, uma vez que avalia a capacidade para essa estirpe penetrar, proliferar e se propagar entre as células, formando placas. A utilização da linha celular HT-29 mostrou-se vantajosa relativamente à utilização da linha celular Caco-2, esta última usada num ensaio PFA (van Langendonck *et al*., 1998).

O sucesso da infecção por *L. monocytogenes* requer a presença e a expressão de genes de virulência, a qual é controlada por várias condições ambientais e pelo hospedeiro, tais como a temperatura, a osmolaridade, o pH e a presença de determinados nutrientes como açúcares e iões (Doyle, 2001; Jaradat e Bhunia, 2003).

1.5. Tipagem molecular

1.5.1. Métodos de tipagem

Vários métodos de tipagem, moleculares e fenotípicos, têm sido usados na diferenciação de estirpes de *Listeria monocytogenes*, nomeadamente: serotipagem (Seeliger e Langer, 1989), fagotipagem (Rocourt *et al*., 1985), *multilocus enzyme electrophoresis* (Bibb *et al*., 1989), *restriction enzyme analysis* (Wesley e Ashton, 1991), *random amplified polymorphic DNA analysis* (Wagner *et al.,* 1996), ribotipagem (Wiedmann *et al*., 1996), *amplified fragment length polymorphism* (AFLP) *analysis* (Guerra *et al*., 2002), *pulsed-field gel electrophoresis* (PFGE) (Graves e Swaminathan, 2001), *multilocus sequence typing* (Revazishvili *et al*., 2004), multi-virulence-locus sequence analysis (Zhang *et al*., 2004) e serotipagem molecular (Doumith *et al*., 2004). Com o objectivo de melhorar a resolução, a rapidez e a reprodutibilidade dos métodos de tipagem de *L. monocytogenes,* novas técnicas estão constantemente a ser introduzidas e testadas. Até à data, a tipagem por PFGE mostrou ser a técnica mais sensível na discriminação de estirpes de *L. monocytogenes* e uma das mais frequentemente empregues.

Seguidamente será explicado o fundamento de cada um dos métodos que foram usados nos capítulos II e III deste trabalho, métodos esses relacionados com a tipagem molecular de estirpes de *Listeria* spp. isoladas de produtos lácteos e do ambiente, e que foram aprendidos e aplicados em Londres no *Department of Gastrointestinal Infections / Health Protection Agency.*

1.5.2. Extracção de DNA

Existem várias formas de obtenção de DNA bacteriano. A tecnologia baseada nas esferas magnéticas e que recorre a um aparelho MagNA Pure LC (Anónimo, 2003) é baseada no método de extracção Boom (Boom *et al*., 1990) e pode ser dividido nas cinco seguintes etapas (Figura 1):

1. Lise do organismo e estabilização do DNA por adição de tampão de lise e de proteinase K;
2. Inactivação do organismo patogénico (opcional), antes da sua transferência para o aparelho MagNA Pure;
3. Ligação do DNA à sílica existente na superfície das partículas magnéticas de vidro em suspensão, devido ao *chaotropic salt condition* e à elevada força iónica do tampão de lise / ligação;
4. Remoção das substâncias não ligadas, como proteínas (nucleases) e constituintes da membrana celular, e de substâncias inibidoras de PCR por passagem em tampões de lavagem;
5. Eluição do DNA purificado a elevadas temperaturas.

O material nuclear obtido por este processo apresenta um elevado grau de pureza e integridade (Anónimo, 2003).

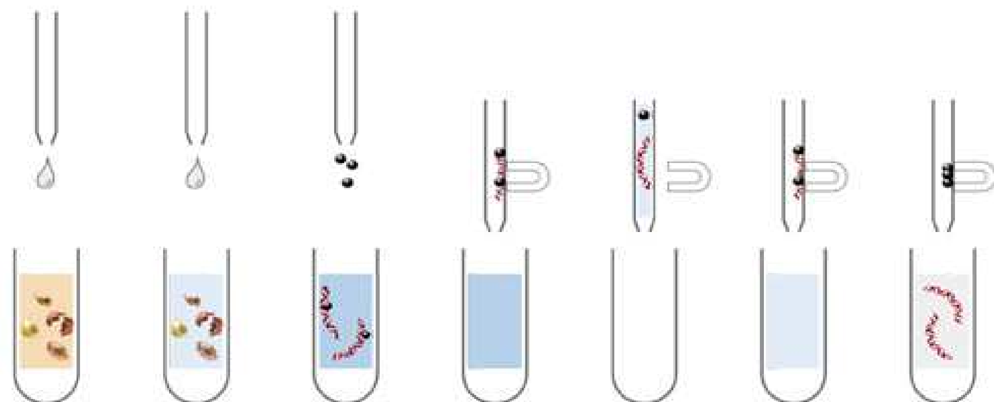

Figura 1. Extracção de DNA através da utilização do aparelho MagNA Pure LC.
Fonte: http://www.roche-applied-science.com/fst/magnapure.htm?/sis/magnapure/products/products_instrument.htm (Acedido em Março de 2007).

1.5.3. *TaqMan Real-Time PCR*

O método *Real Time - Polymerase Chain Reaction* (RT-PCR) foi introduzido recentemente e combina a amplificação de DNA com a detecção dos seus produtos num simples tubo, reduzindo-se significativamente o risco de contaminação causado

pela abertura dos tubos durante a manipulação pós-PCR. Para além desta vantagem, é mais rápido do que a análise baseada nos géis, pode fornecer resultados quantitativos e pode ser acompanhado em tempo real através da monitorização da fluorescência durante cada ciclo de PCR (Eurogentec, 2004).

As sondas TaqMan® (também designadas por *double-dye oligonucleotide* ou *dual labelled probes*) são as sondas para RT-PCR mais amplamente usadas. Estas sondas têm um fluoróforo numa extremidade e um *quencher* na outra. Quando o fluoróforo é excitado pela máquina, passa a energia via *Fluorescent Resonant Energy Transfer* (FRET), para o *quencher*. A sonda TaqMan está localizada entre os dois *primers* PCR e tem uma temperatura de fusão 5 a 10 ºC superior à dos *primers*, de forma a que a ligação da sonda TaqMan se efectue antes da ligação dos *primers*, o que é fundamental para que os produtos do PCR sejam formados com a geração de uma intensa fluorescência de forma a serem detectados (Kendall *et al*., 2000).

Considerando a utilização do método *TaqMan Real-Time* PCR na detecção de *Listeria monocytogenes*, o gene mais vulgarmente usado como alvo é o *hlyA*, responsável pela expressão do factor de virulência listeriolisina O (Tabela 2). Mais concretamente, é feita a detecção de um fragmento deste gene, o qual apresenta 113 bp (Novga *et al*., 2000).

Tabela 2. *Primers* e sonda fluorogénica específica para a detecção do gene de *L. monocytogenes* que codifica para o factor de virulência listeriolisina O.

Gene alvo	Sonda / *Primer*	Sequência (5´- 3´)
	hlyA sonda	CGA TTT CAT CCG CGT GTT TCT TTT CG
hlyA	*hlyA-For primer*	TGC AAG TCC TAA GAC GCC
	hlyA-Rev primer	CAC TGC ATC TCC GTG GTA TAC TAA

For – *Forward*; Rev – *Reverse*.

Fonte: Novga *et al*. (2000).

1.5.4. *Amplified fragment length polymorphism*

A técnica *amplified fragment length polymorphism* (AFLP), ou "polimorfismo de fragmentos de restrição amplificados", inicialmente desenvolvida por Vos *et al*. (1995) para a análise do genoma de plantas, já foi usada com sucesso na caracterização de bactérias e de fungos (Janssen *et al*., 1996). Baseia-se na amplificação selectiva de fragmentos de DNA previamente obtidos a partir da digestão da totalidade do genoma bacteriano. O DNA extraído e purificado é digerido com uma ou duas enzimas de restrição e cada fragmento resultante vai ser ligado, nas duas extremidades de restrição, a adaptadores oligonucleotídicos de cadeia dupla, aos quais se vai depois ligar um *primer* específico (Gibson *et al*., 1998). Os adaptadores são desenhados de forma a interromperem a acção da enzima de restrição, bloqueando os respectivos locais de corte e evitando desta forma novas digestões (Guerra *et al*., 2002). Dado que os adaptadores não são fosforilados, não ocorre a ligação adaptador-adaptador (Olive e Bean, 1999). A amplificação selectiva dos fragmentos numa reacção de polimerização em cadeia (PCR) é conseguida através do uso de um *primer* complementar da sequência de bases contíguas no adaptador e no local de restrição, mais um ou dois nucleótidos do DNA alvo original na extremidade 3´. Os fragmentos de DNA amplificados por PCR são depois separados por electroforese em gel de agarose corado com brometo de etídio, e os perfis obtidos são visualizados com luz UV e fotografados para posterior análise (Gibson *et al*., 1998; McLauchlin *et al*., 2000).

A técnica AFLP conjuga o poder da análise por *restriction fragment length polymorphism* (RFLP), através do uso de enzimas de restrição, com a flexibilidade da tecnologia baseada em PCR, gerando um número de fragmentos suficientemente grande para permitir a detecção de polimorfismos entre indivíduos pertencentes à mesma espécie e a estirpes estritamente relacionadas, mas suficientemente pequeno para ser facilmente interpretável. Para além destas vantagens, a técnica AFLP é rápida, altamente reprodutível, tem elevada resolução e não requer o conhecimento prévio das sequências genómicas.

A aplicação da técnica AFLP à tipagem molecular de *L. monocytogenes* já provou ser útil (Guerra *et al*., 2002; Autio *et al*., 2003; Vogel *et al*., 2004),

apresentando as vantagens de ser mais rápida e menos dispendiosa do que a técnica PFGE.

Na figura seguinte encontram-se as sequências para o *primer* *Eco*-R1G e os adaptadores *Eco* AD1 e *Eco* AD2. A enzima de restrição *Eco*RI, muito usada em AFLP, corta o DNA nos locais de restrição 5´-G↓AATTC-3´ e 3´-CTTAA↓G-5´.

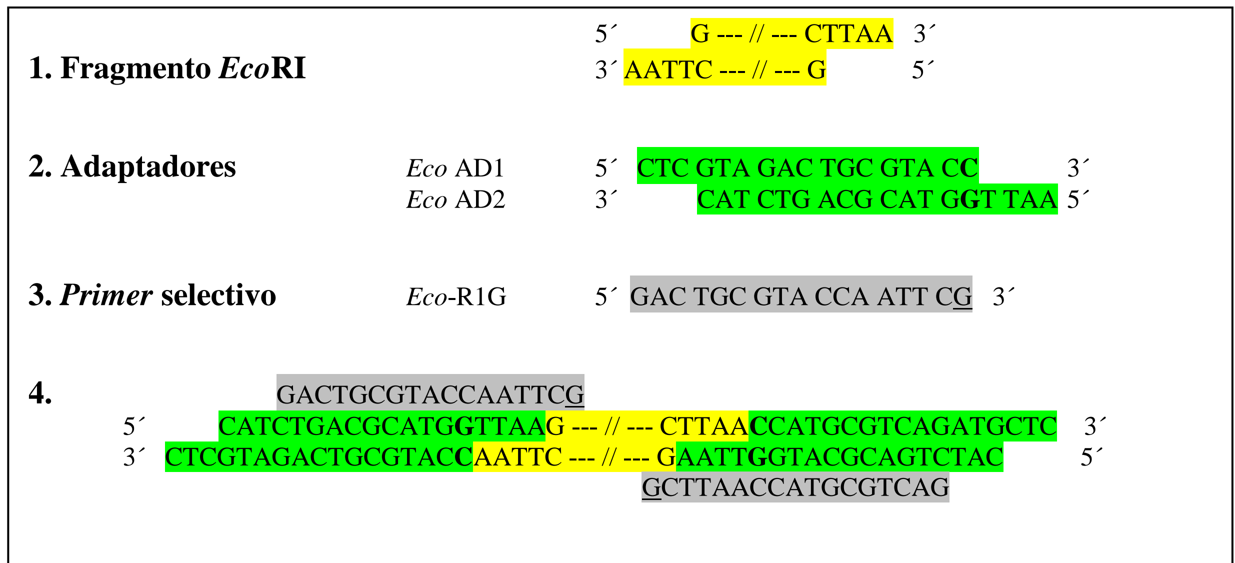

Figura 2. Local de restrição da *Eco*RI e sequências nucleotídicas dos adaptadores e do *primer* específico usado, na tipagem de *Listeria monocytogenes* por AFLP.

(1) Fragmento produzido pela digestão com EcoRI (com local de corte G↓CTTAA). **(2)** Sequências dos dois oligonucleótidos complementares que constituem o adaptador que se vai ligar a cada extremidade de cada segmento de restrição. Os nucleótidos a negrito são bases inseridas no adaptador para eliminar a restrição deste local após ligação. **(3)** Sequência nucleotídica do *primer* usado na fase de amplificação. G é a base selectiva inserida no *primer*. **(4)** Fragmento pronto a ser amplificado numa reacção PCR.

1.5.5. Serotipagem molecular

A serotipagem de *L. monocytogenes* tem vindo a ser usada como método de subtipagem há décadas, permitindo diferenciar 13 diferentes serovares com base nas diferentes combinações entre os antigénios somáticos O (nos ácidos teicóicos da parede celular) e os antigénios flagelares H (Seeliger e Höhne, 1979) (Tabela 3).

Esta é uma técnica simples, muitas vezes usada para efectuar uma segregação preliminar das estirpes em estudo antes da aplicação de métodos de tipagem mais discriminatórios, apesar da reprodutibilidade nem sempre ser satisfatória (Schönberg *et al*., 1996). No entanto, a serotipagem tradicional com os antisoros factor O e factor H está implementada apenas num pequeno número de laboratórios de referência (Nadon *et al*., 2001) e estão descritas reacções cruzadas com outras bactérias Gram-positivas, nomeadamente *Enterococcus* spp. e *Staphylococcus aureus*, e com algumas estirpes de *Escherichia coli* (Seeliger e Höhne, 1979). Para além destes aspectos, à serotipagem atribui-se um fraco poder discriminatório, o qual se traduz em: (1) a quase totalidade das estirpes de *L. monocytogenes* isoladas quer de alimentos quer de material patológico pertencem a um reduzido número de serovares (serovares 4b, 1/2a e 1/2b) (McLauchlin, 1987; Rocourt e Jacquet, 1993); (2) as espécies *L. monocytogenes* e *L. seeligeri* são antigenicamente idênticas entre si (Seeliger e Langer, 1989); (3) as reacções ao antisoro factor H são em geral mais fracas do que as do antisoro factor O, tornando-se a interpretação dos resultados mais subjectiva, especialmente para os serovares 1/2c e 3c; (4) quando as estirpes não são móveis, estas podem não ser totalmente tipáveis devido à falta dos antigénios flagelares H, os quais são importantes para a diferenciação das estirpes dentro do serogrupo 1/2 (Palumbo *et al*., 2003).

Tabela 3. Antigénios dos serovares de *L. monocytogenes*.

Serovar	Antigénios O (antigénios somáticos)	Antigénios H (antigénios flagelares)
1/2a	I, II, (III)	A, B
1/2b	I, II, (III)	A, B, C
1/2c	I, II, (III)	B, D
3a	II, (III), IV	A, B
3b	II, (III), IV, (XII), (XIII)	A, B, C
3c	II, (III), IV, (XII), (XIII)	B, D
4a	(III), (V), VII, IX	A, B, C
4ab	(III), V, VI, VII, IX, X	A, B, C
4b	(III), V, VI	A, B, C
4c	(III), V, VII	A, B, C
4d	(III), (V), VI, VIII	A, B, C
4e	(III), V, VI, (VIII), (IX)	A, B, C
7	(III), XII, XIII	A, B, C

( ) Os antigénios dentro de parêntesis podem não estar presentes em todos os isolados.

Fonte: Adaptado de Seeliger e Jones (1987)

De forma a minimizar algumas das desvantagens da aglutinação tradicional, referidas acima, têm vindo a ser implementados métodos alternativos baseados nos caracteres moleculares (Palumbo *et al.,* 2003; Borucki e Call, 2003; Doumith *et al.*, 2004).

No *Institut Pasteur* de *Paris*, Doumith *et al*. (2004) desenvolveram um novo ensaio PCR *multiplex*, o qual permite separar os quarto serovares de *L. monocytogenes* mais frequentes (1/2a, 1/2b, 1/2c, 4b) em quatro grupos distintos (Figura 3). Os genes marcadores seleccionados para esta técnica são *lmo0737*, *lmo1118*, *ORF2819*, *ORF2110* e *prs*. O gene *prs* é específico para o género *Listeria*. As características dos *primers* referidos acima bem como a respectiva especificidade encontram-se na tabela 4.

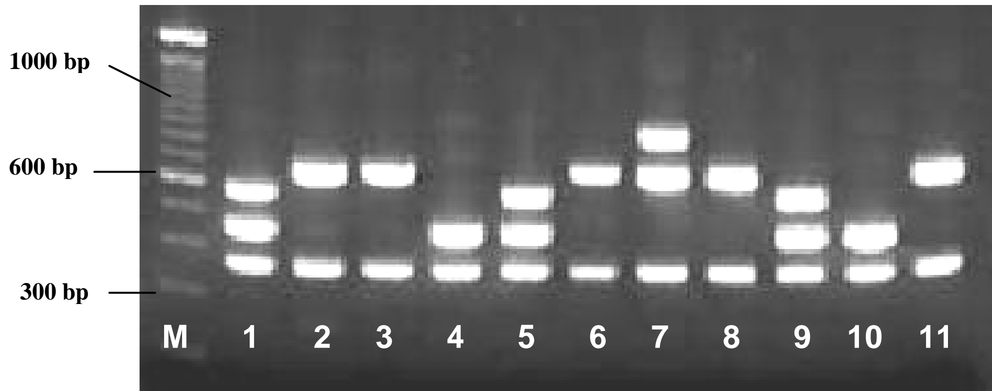

Figura 3. Serotipagem molecular de *Listeria monocytogenes*, de acordo com a técnica descrita por Doumith *et al*. (2004).

M – Marcador; Perfis 1, 5 e 9 - serogrupo 4b, 4d, 4e; Perfis 2, 3, 6, 8 e 11 - serogrupo 1/2a, 3a; Perfis 4 e 10 - serogrupo 1/2b, 3b, 7; Perfil 7 - serogrupo 1/2c, 3c.

Fonte: Pintado (2005).

Tabela 4. Sequências nucleotídicas e características dos *primers* usados na serotipagem molecular.

Gene alvo	*Primer* (5´-3´)	Product size (bp)	Especificidade
Lmo0737	**For:** AGG GCT TCA AGG ACT TAC CC	691	*L. monocytogenes* serovares
	Rev: ACG ATT TCT GCT TGC CAT TC		1/2a, 1/2c, 3a, 3c
Lmo1118	**For:** AGG GGT CTT AAA TCC TGG AA	906	*L. monocytogenes* serovares
	Rev: CGG CTT GTT CGG CAT ACT TA		1/2c, 3c
ORF2819	**For:** AGC AAA ATG CCA AAA CTC GT	471	*L. monocytogenes* serovares
	Rev: CAT CAC TAA AGC CTC CCA TTG		1/2b, 3b, 4b, 4d, 4e
ORF2110	**For:** AGT GGA CAA TTG ATT GGT GAA	597	*L. monocytogenes* serovares
	Rev: CAT CCA TCC CTT ACT TTG GAC		4b, 4d, 4e
prs	**For:** GCT GAA GAG ATT GCG AAA GAA G	370	*Listeria* spp.
	Rev: CAA AGA AAC CTT GGA TTT GCG G		

For – *Forward*; Rev – *Reverse*.

Fonte: Doumith *et al*. (2004)

1.5.6. *Pulsed-field gel electrophoresis*

A análise de DNA por *pulsed-field gel electrophoresis* (PFGE) é geralmente considerada a *gold standard* dos métodos de tipagem molecular (Olive e Bean, 1999), tendo vindo a ser aplicada com sucesso à tipagem de *L. monocytogenes* (Miettinen *et al*., 1999; Lundén *et al*., 2002; Sauders *et al*., 2003; Wagner *et al*., 2003; Fugett *et al*., 2007; Chambel *et al*., 2007).

A preparação do DNA para a macrorestrição genómica baseia-se no princípio da lise das células inteiras previamente incorporadas em agarose, de acordo com uma pequena alteração da metodologia usada por Moore e Datta desde 1994 (Dauphin *et al*. 2001). O DNA cromossómico é digerido com endonucleases de restrição que cortam pouco frequentemente. A electroforese é efectuada num aparelho próprio, no qual a polaridade da corrente é alterada a intervalos predefinidos, o que permite a separação de fragmentos de DNA de peso molecular muito elevado e a produção de perfis simples. A comparação entre os perfis de diferentes estirpes torna-se assim simples e rápida.

A rede de laboratórios americana *PulseNet* desenvolveu um protocolo para *L. monocytogenes* (Graves e Swaminathan, 2001) que recorre à utilização de duas endonucleases de restrição, *AscI* e *ApaI,* as quais produzem fragmentos com a mesma amplitude de pesos (Kb). A utilização da *AscI* e da *ApaI* com um conjunto de estirpes certificadas permitiu identificar 8 a 14 e 14 a 19 bandas, respectivamente.

Na figura 4 podemos ver um esquema que resume as principais etapas envolvidas na tipagem por PFGE.

Com o objectivo de uniformizar a interpretação dos perfis de PFGE relativamente à semelhança entre diferentes estirpes, Tenover *et al*. (1995) propuseram critérios aplicáveis a pequenos estudos localizados em que se presume que a variabilidade genética é pequena.

Um dos factores que limitou no passado o uso da análise de DNA por PFGE foi o tempo necessário para uma análise completa, 3 dias. Para responder a esta limitação, Graves e Swaminathan (2001) propuseram um protocolo mais rápido, de cerca de 30 horas.

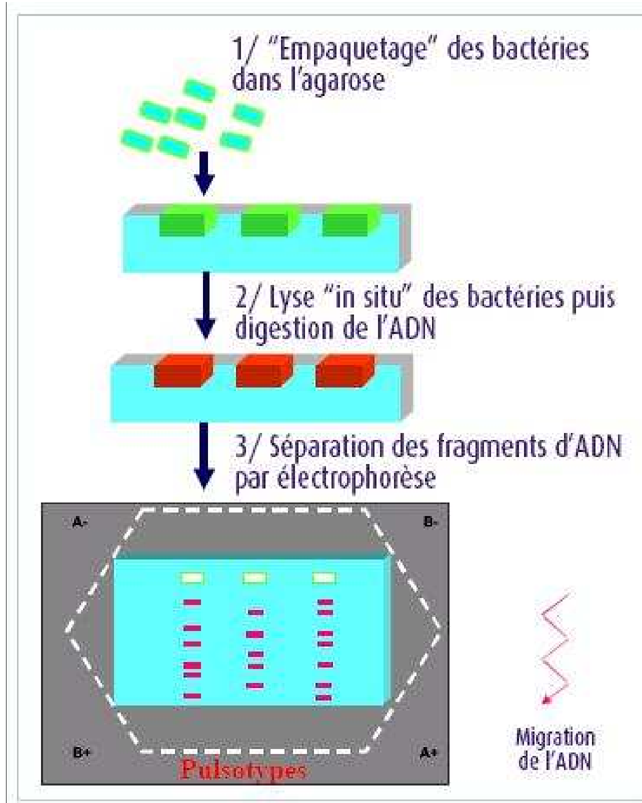

Figura 4. Tipagem molecular por PFGE.

(1) Incorporação das células inteiras em agarose (*plots*); **(2)** Lise das células *in situ* e digestão do DNA com endonucleases de restrição; **(3)** Separação dos fragmentos de DNA por aplicação de electroforese em campo pulsado.

Fonte: http://www.pasteur-lille.fr/fr/expertises/alimentaire/techniques/electrophorese_mars.html. (Acedido em Março de 2007)

1.6. Sensibilidade de *Listeria* spp. a nisina

A nisina (E234) é um peptídeo composto por 34 amino-ácidos. É uma bacteriocina pertencente ao grupo dos lantibióticos devido à presença dos amino-ácidos invulgares, lantionina e β-metil lantionina (Sen *et al.*, 1999). Há duas variantes naturais da nisina, a nisina A e a nisina Z, as quais são produzidas pela bactéria láctica *Lactococcus lactis* ssp. *lactis* (Bauer e Dicks, 2005). Estas duas variantes diferem apenas no resíduo de amino-ácido localizado na posição 27 (Figura

5). A nisina é representada pela fórmula $C_{143}H_{230}N_{4}2O_{37}S_{7}$ e tem um peso molecular de 3354 Daltons. Esta molécula é solúvel em água e outros solventes polares.

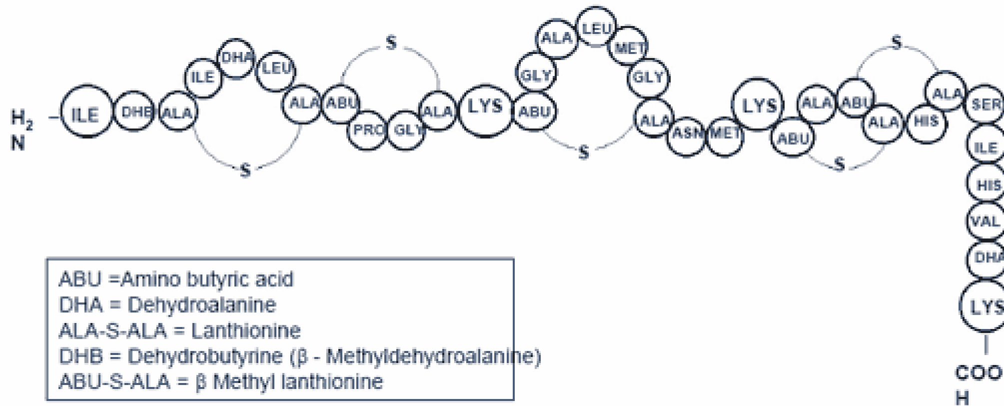

Figura 5 – Estrutura da molécula de nisina A.

A nisina Z apresenta asparigina em vez de histidina na posição 27.

Fonte: http://en.engormix.com/images/e_articles/822_01.gif (Acedido em Maio de 2009)

Em 1995, foi autorizada a utilização de nisina como conservante alimentar em queijo curado e queijo processado, de acordo com a directiva comunitária 95/2/EC, na quantidade de 12,5 mg/Kg (Anónimo, 1995). Em 2001, a *Food and Drug Administration* reconheceu o estatuto GRAS (*generally recognised as safe*) para ser usada como agente antimicrobiano em produtos cárneos (FDA, 2001).

Quanto ao seu espectro de inibição, a nisina tem um efeito antimicrobiano contra esporos e células vegetativas de bactérias Gram-positivas (*Bacillus, Clostridium, Listeria monocytogenes*). Na ausência de outros agentes antimicrobianos, não é eficaz contra bactérias Gram-negativas, bolores ou leveduras (Bauer e Dicks, 2005). Para que o seu espectro de acção seja mais alargado, recomenda-se a utilização conjunta da nisina com outros métodos ou agentes, como a diminuição do pH e o aumento da concentração de sais (Ariyapitipun *et al.*, 1999). A adição de Tween 80, um emulsionante não iónico, a amostras de leite aumentou significativamente a actividade de nisina sobre o crescimento de *Listeria monocytogenes* (Jung *et al.*, 1992).

A nisina liga-se aos fosfolípidos aniónicos (incluindo lípidos II) da membrana celular (Bonev *et al.*, 2004; Kramer *et al.*, 2004), levando à formação de poros, ao efluxo dos componentes intracelulares e à deplecção da força proto-motriz. Em consequência da despolarização da membrana citoplasmática, a biossíntese cessa. Para além destes efeitos, a ligação da nisina aos lípidos II conduz à inibição da biossíntese do peptidoglicano, componente da parede celular.

Os microrganismos que apresentam resistência a nisina têm uma via enzimática que inactiva o peptideo ou, em alternativa, apresentam alterações na susceptibilidade da membrana citoplasmática. Por exemplo, *Lactobacillus plantarum* produz a enzima nisinase que neutraliza a nisina (Hurst e Hoover, 1993). Por outro lado, têm sido relatados mutantes espontâneos resistentes a nisina em microrganismos como *L. monocytogenes*. Relativamente a esta bactéria, a literatura refere que os mutantes resistentes a nisina são estáveis e podem ocorrer a uma taxa de 1 em 10^6 ou 10^8. As estirpes de *L. monocytogenes* resistentes a nisina apresentam alterações na composição dos fosfolípidos da membrana citoplasmática, incluindo uma diminuição dos fosfolípidos aniónicos, resultando numa diminuição da carga negativa líquida, limitando desta forma a possibilidade de ligação à nisina, que é catiónica (Crandall e Montville, 1998). Verifica-se também que a membrana citoplasmática das estirpes de *L. monocytogenes* resistentes a nisina exibem um aumento do número de ácidos gordos de cadeia longa e uma redução da proporção entre os ácidos gordos em C15 relativamente aos ácidos gordos em C17, o que sugere uma redução na fluidez e estabilização causada pelo efeito reduzido na força proto-motriz. Mazzotta *et al.* (2000) verificaram que as estirpes de *L. monocytogenes* resistentes a nisina apresentavam maior sensibilidade a outros agentes antimicrobianos, por comparação com as estirpes selvagens.

A resistência a nisina não parece ser transmissível entre microrganismos. Apesar do uso de nisina na indústria alimentar já se verificar há alguns anos, não há indicações de que a resistência tenha aumentado, o que pode ser devido ao duplo modo de acção da nisina (Kramer *et al.*, 2004).

2. Revestimentos edíveis com propriedades antimicrobianas

2.1. Características gerais dos revestimentos

A embalagem de produtos alimentares deve prolongar a vida útil dos produtos, mantendo as suas características físicas, químicas e organolépticas pelo tempo requerido, evitando ou minimizando as perdas. A embalagem activa é um caso particular de embalagem que responde a alterações que ocorrem no produto ou na sua envolvência e como exemplo temos as embalagens com propriedades antimicrobianas (Poças e Moreira, 2003). Nesta definição estão englobados os filmes e os materiais ou revestimentos que após secagem envolvem o produto alimentar.

Filmes ou revestimentos são matrizes contínuas que podem ser preparadas a partir de proteínas, polissacáridos ou lípidos (Cagri *et al.*, 2004).

As proteínas são polímeros naturais totalmente biodegradáveis. Embora a sua estrutura não contenha motivos regulares repetitivos têm sido realizados consideráveis esforços de forma a explorar e desenvolver filmes à base de proteínas. As proteínas que têm vindo a ser estudadas com esse objectivo são o colagénio, a zeína de milho, o glúten de trigo, o isolado proteico de soja, o isolado proteico de soro de leite e a caseína (Alves e Tomás, 2003).

Os plastificantes são componentes importantes da formulação dos revestimentos e filmes, os quais aumentam a flexibilidade devido à sua capacidade para reduzir as ligações de hidrogénio internas entre as cadeias do biopolímero, aumentando a mobilidade e o espaço intermolecular e reduzindo a rigidez (McHugh *et al.*, 1994). O plastificante mais eficaz é aquele cuja estrutura se assemelha mais com a estrutura do polímero que ele plastifica (Mali *et al.*, 2005). O efeito plastificante aumenta com a diminuição do peso molecular e o aumento da hidrofobicidade do agente plastificante (Cagri *et al.*, 2001). Os grupos hidroxilo do plastificante vão substituir as interacções polímero-polímero estabelecendo-se ligações de hidrogénio entre o polímero e o plastificante. O tipo e concentração de plastificante influenciam as propriedades dos filmes e revestimentos, nomeadamente a permeabilidade ao vapor de água e a gases. Tal como as propriedades de barreira, também a resistência mecânica e a elasticidade diminuem com o aumento da concentração de plastificante (Cagri *et al.*, 2004). O sorbitol, o glicerol, o manitol, a

sacarose e o polietileno-glicol são exemplos de agentes plastificantes. Também os ácidos orgânicos podem ter um efeito plastificante, dado serem moléculas pequenas com grupos hidroxilo. O glicerol, com um peso molecular de 92 g/mol (Zielinski, 1997), é um dos agentes plastificantes mais usados.

2.2. Revestimentos à base de isolado proteico de soro de leite

As proteínas do soro do leite são a fracção proteica que permanece solúvel após a precipitação da caseína a pH 4,6, são de natureza globular e constituem 20% do total de proteínas do leite. As principais proteínas do soro de leite são a β-lactoglobulina (β-Lg; 50-75%), a α-lactoalbumina (25%), a albumina do soro bovino, as proteases-peptonas e as imunoglobulinas (Krochta, 1997). A β-lactoglobulina contém dois grupos bissulfito, um grupo livre sulfídrico (grupo tiol) e grupos hidrofóbicos localizados no interior da estrutura globular (Krochta, 1997). Enquanto a β-lactoglobulina tem 162 resíduos de amino-ácidos e uma massa molecular de 18,3 KDa, a α-lactoalbumina é uma molécula mais pequena, de 123 amino-ácidos e 14,2 KDa e constituída por quatro pontes bissulfito (Cayot e Lorient, 1997) (Tabela 5).

Podem ser encontradas duas formas comerciais de proteína de soro de leite, o concentrado proteico de soro (WPC, *whey protein concentrate*) com 25-80% de teor proteico e o isolado proteico de soro (WPI, *whey protein isolate*) com um teor proteico superior a 80% (Krochta, 1997). De acordo com Banerjee e Chen (1995), as propriedades funcionais de filmes elaborados com WPI e WPC são idênticas; no entanto, a utilização de WPC é aconselhável visto ser a solução mais económica.

As propriedades físico-químicas dos amino-ácidos, como a carga, solubilidade e reactividade química dependem da natureza química do grupo funcional. A carga líquida de uma proteína a um determinado valor de pH é determinada pelo número relativo de resíduos de amino-ácido básicos (Arg, Lys e His) e resíduos ácidos (Glu e Asp) na proteína (Damodaran, 1997).

Um dos principais factores que influenciam as propriedades das proteínas como a estabilidade conformacional, a solubilidade, a actividade de superfície e a ligação a gorduras é a hidrofobicidade geral dos resíduos dos amino-ácidos constituintes (Tabela 6). Os amino-ácidos funcionais com valores de ΔG_t positivos e

elevados são hidrofóbicos (localizam-se preferencialmente na fase orgânica ou no interior da proteína). Os amino-ácidos funcionais com valores de ΔG_t negativos localizam-se preferencialmente na parte exterior da proteína em contacto com o meio aquoso (Damodaran, 1997).

Tabela 5. Características físico-químicas das principais proteínas do soro de leite.

Características	β-Lactoglobulina	α-Lactoalbumina	Albumina bovina do soro	Imunoglobulinas
Massa molar (g / mol)	18,362	14,174	69	150 000-1 000 000
Resíduos de cisteína / mol	5	8	35	___
Resíduos de amino-ácidos / mol	162	123	582	>1000
Ligações bissulfito / mol	2	4	17	4.x
Função tiol / mol	1	0	1	___
Resíduos de lisina/ mol	15	12	59	___
Resíduos de arginina / mol	3	1	23	
Resíduos de histidina / mol	2	3	18	
Resíduos de glutamina / mol	16	8	59	___
Resíduos de aspartato / mol	10	9	39	___
PI (ponto isoeléctrico)	5.2	4.5-4.8	4.7-4.9	5.5-8.8
Hidrofobicidade média (kJ/resíduo)	508	468	468	458

Fonte: Cayot e Lorient (1997)

Devido à natureza globular das proteínas do soro de leite, a produção de filmes requer uma desnaturação térmica para abrir a estrutura globular da proteína e quebrar as ligações bissulfito existentes, estabelecendo-se novas ligações intermoleculares do tipo bissulfito e ligações hidrofóbicas (McHugh *et al.*, 1994). Os filmes resultantes são transparentes e frágeis, necessitando da incorporação de um agente plastificante para os tornar mais extensíveis e menos rígidos ou quebradiços. A produção de filmes proteicos de soro de leite também se faz recorrendo à utilização de transglutaminases, mas o seu elevado custo limita a sua aplicação em alimentos (Krochta, 1997).

Tabela 6. Características dos amino-ácidos da principal proteína do leite de vaca.

Amino-ácido	Composição em amino-ácidos (% resíduos / Mol proteina) da principal proteína do soro de leite		Solubilidade em água	Hidrofobicidade dos amino-ácidos funcionais a 25 ºC
	Isolado proteico de soro	β-Lactoglobulina A		(ΔG_t (etanol →água), KJ/mol)
Lisina	8,3	9,2	(1)	___
Histidina	7,8	1,2	(1)	2,09
Arginina	1,9	1,8	(1)	___
Ácido aspártico	10,0	9,9	(1)	2,09
Treonina	4,7	4,9	(2)	1,67
Serina	7,9	4,3	(2)	- 1,25
Ácido glutâmico	13,3	15,4	(1)	2,09
Prolina	4,9	4,9	(3)	10,87
Glicina	2,6	1,8	(2)	0
Alanina	6,6	8,6	(3)	2,09
Cisteína/2	2,4	3,1	(2)	4,18
Valina	4,0	6,2	(3)	6,25
Metionina	1,6	2,5	(3)	5,43
Isoleucina	4,7	6,2	(3)	12,54
Leucina	12,3	13,6	(3)	9,61
Tirosina	2,5	2,5	(4)	9,61
Fenilalanina	2,5	2,5	(4)	10,45
Triptofano	1,2	1,2	(4)	14,21
Asparagina			(2)	0
Glutamina				- 0,42

(*) Resíduo de amino-ácido básico ; (*) Resíduo de amino-ácido ácido ; **(1)** Grupo funcional carregado / Polar / Solúvel em água; **(2)** Grupo funcional não carregado / Polar / Solúvel em água; **(3)** Grupo funcional alifático / Não polar / Não solúvel em água; **(4)** Grupo funcional aromático / Não polar / Não solúvel em água.

Fonte : Gennadios *et al*. (1994) e Damodaran (1997).

A formação de filmes de proteína de soro de leite é favorecida em soluções mais alcalinas uma vez que a reactividade dos grupos SH aumenta a pH superior a 8 (Cagri *et al*, 2001). Em situações de baixo pH as ligações S-S na matriz proteica são muito provavelmente inibidas, enfraquecendo a estrutura do filme. Por este motivo, a

resistência mecânica destes filmes é inferior à dos filmes com pH mais elevado (McHugh e Krochta, 1994).

De acordo com Kella e Kinsella (1988), a pH 7,5 a β-lactoglobulina está carregada negativamente relativamente ao pI (pH 5,2), apresentando sete cargas negativas líquidas por monómero (por comparação com o pI) a pH 7,5 e apenas quatro a pH 6,5. Esta situação pode facilitar o empacotamento dos grupos no interior da proteína, o que aumenta as interacções hidrofóbicas no interior da proteína e aumenta também as atracções de van der Waals. Quando o pH diminui abaixo do pI, a carga positiva líquida da proteína aumenta, sendo de quinze unidades por monómero a pH 3,0 e dezoito a pH 1,5. Seria previsível que esta situação levasse à desestabilização da proteína devido à repulsão mútua e enfraquecimento das interacções de van der Waals. No entanto, verifica-se um aumento da estabilidade da β-lactoglobulina a pH baixo. Este aumento da estabilidade pode ser atribuído às ligações de H adicionais devido à neutralização dos grupos carboxilo. Kella e Kinsella (1988) verificaram também que a temperatura de desnaturação (*Tm*) da β-lactoglobulina aumentava com a diminuição do pH (77,2ºC a pH 3,0 e 78,8ºC a pH 2,5), sugerindo um aumento da estabilidade molecular.

O pH é um factor que influencia fortemente a estrutura dos géis elaborados com β-lactoglobulina (Otte *et al.*, 2000; Yoshida e Antunes, 2004). Segundo Otte *et al.* (2000), os géis de β-lactoglobulina com pH 7,0 são compostos por polímeros ligados internamente por ligações covalentes (ligações bissulfito), e associados principalmente por forças não covalentes (interacções hidrofóbicas e/ou pontes de H), compensadas por repulsões electrostáticas negativas. A pH 3,0 a matriz do gel parece ser mantida principalmente por forças de atracção não covalentes (pontes de H e interacções hidrofóbicas), compensadas por interacções repulsivas electrostáticas. Ainda segundo os mesmos autores, a pH próximo do pI da proteína a carga eléctrica é mínima e dominam as interacções atractivas hidrofóbicas, complementadas por interacções electrostáticas locais.

2.3. Revestimentos com propriedades antimicrobianas

Para além de propriedades como a biodegradabilidade, edibilidade e propriedades de barreira a gases e vapor de água, os revestimentos podem ser usados como matrizes para a incorporação de agentes antimicrobianos e de conservantes. Vários têm sido os agentes usados com este propósito, tais como: nisina, lisozima, ácidos orgânicos, natamicina, sorbato de K, ácido para-aminobenzóico.

A incorporação destes agentes antimicrobianos nos filmes ou revestimentos pode ser encarada como uma nova forma de aumentar a segurança alimentar dos alimentos assim revestidos na medida em que: (1) diminui o risco de transmissão de agentes patogénicos, (2) previne alterações na superfície dos alimentos e (3) aumenta a sua validade.

Os revestimentos com propriedades antimicrobianas previnem o crescimento microbiano por contacto directo do revestimento com a superfície do alimento. A libertação gradual dos agentes para a superfície do alimento é vantajosa relativamente à sua aplicação por imersão numa solução antimicrobiana não filmogénica, uma vez que neste último caso a actividade antimicrobiana pode ser perdida ou reduzida devido a interacções com componentes do alimento, como no caso da gordura (Jung *et al*., 1992), ou a migração para o seu interior (Appendini e Hotchkiss, 2002). A taxa de difusão dos agentes antimicrobianos incorporados nos filmes ou revestimentos de proteína de soro pode ser controlada através da alteração do pH e da proporção entre proteína de soro e plastificante, uma vez que a carga das proteínas e o tamanho dos poros na matriz proteica tridimensional dependem daqueles factores (Han, 2000). O método usado na preparação dos filmes proteicos também vai influenciar a difusão do agente para o alimento (Teerakarn *et al*., 2002).

Vários ácidos orgânicos têm sido usados nas formulações dos filmes bioactivos, como o acético, cítrico, succinico, málico, tartárico, benzóico e sórbico. Eswaranandam *et a*l. (2004) estudaram a incorporação dos ácidos málico, cítrico, láctico e tartárico em filmes de proteína de soja e verificaram que o ácido málico foi o que apresentou melhores capacidades na inibição de *Listeria monocytogenes*, *Salmonella* e *Escherichia coli* O157:H7.

A nisina é um dos agentes mais estudados na produção de filmes antimicrobianos e vários trabalhos têm mostrado a sua eficácia na inibição de

bactérias Gram-positivas, nomeadamente *Listeria monocytogenes,* mas também, quando coadjuvada com um agente quelante, na inibição de bactérias Gram-negativas (Ko *et al*., 2001; Janes *et al*., 2002; Eswaranandam *et a*l., 2004; Hoffman *et al*., 2001; Teerakarn *et al*., 2002). Ko *et al*. (2001) estudaram o efeito da incorporação de nisina em filmes preparados com diferentes tipos de proteína (soro de leite, trigo, soja e milho) relativamente à actividade inibitória contra *Listeria monocytogenes* e bactérias lácticas, tendo concluído que os filmes de proteína de soro de leite com nisina foram produziam halos de inibição maiores. Segundo estes autores, isto deve-se ao facto da nisina apresentar maior actividade em meios hidrofóbicos, e de os filmes de proteína de soro de leite serem os que têm um maior número de resíduos hidrofóbicos, comparativamente às restantes proteínas estudadas, apresentando maior hidrofobicidade.

3. Enquadramento geral e objectivos

Como já foi referido anteriormente, *L. monocytogenes* é um microrganismo patogénico de origem alimentar, capaz de provocar casos graves de listeriose em indivíduos YOPI (*young, old, pregnant and immunocompromised*), provocando uma taxa de mortalidade média de 20-30%. Os produtos lácteos em geral têm sido frequentemente implicados na transmissão do agente etiológico da listeriose (Dauphin *et al*., 2001). De particular importância temos os queijos de pasta mole ou semi-mole, elaborados com leite cru e que são durante o seu processo de fabrico muito manipulados. Segundo ILSI (2005), o queijo encontra-se na categoria dos alimentos de alto risco, visto que: (1) tem potencial para ser contaminado por *L. monocytogenes*; (2) suporta o crescimento de *L. monocytogenes* em elevados números; (3) é um alimento pronto-a-comer; (4) requer refrigeração; (5) é conservado durante longos períodos.

Apesar de não haver, até hoje, registos da ocorrência de surtos de listeriose em Portugal, a elevada ocorrência de *Listeria monocytogenes* em queijos no nosso país (Duarte, 1992; Pintado, 1996; Guerra e Bernardo, 1997) leva-nos a considerar estes produtos como de elevado risco, justificando todos os esforços que se fizeram, e possam vir a fazer, no sentido de conhecer a sua ocorrência e possível persistência nas queijarias, a complexidade das vias de contaminação e a forma mais adequada para o seu controlo. Em Portugal, a listeriose não é uma doença de notificação obrigatória e o Programa de Vigilância e Controlo das Toxinfecções Alimentares Colectivas não foi ainda implementado com sucesso, o que significa que não conhecemos a real situação do nosso país nesta matéria.

Portugal é um país com uma notável tradição na produção de queijos artesanais típicos de várias regiões. O Queijo de Castelo Branco e o Queijo de Tolosa, produzidos respectivamente na Sub-Região Demarcada do Queijo de Castelo Branco e na Região Demarcada do Queijo de Tolosa, são dois desses exemplos, sendo produzidos com leite cru de ovelha *in estreme* e coalhados com uma infusão de cardo.

O trabalho de doutoramento aqui apresentado vem na sequência do trabalho desenvolvido no mestrado, intitulado "Isolamento de *Listeria monocytogenes* no Queijo de Castelo Branco usando o método IDF e o Listeria-Tek ELISA".

Considerando os dados obtidos, verificou-se que a contaminação de queijo de ovelha por *Listeria monocytogenes* era muito elevada, exigindo um estudo detalhado sobre as possíveis vias de contaminação e formas de controlo. Desta forma, o plano do doutoramento agora apresentado, e intitulado "Efeito de bioconservantes no crescimento e sobrevivência de *Listeria monocytogenes* em queijo de ovelha", pretende contribuir para a resolução do problema associado à presença desta bactéria patogénica em produtos típicos, com denominação de origem protegida (DOP), e com elevado peso económico na região, como é o caso do Queijo de Castelo Branco. O cumprimento deste objectivo geral, relacionado com o aumento da segurança alimentar no consumo de queijos produzidos com leite cru de ovelha, implicou a organização do presente trabalho em duas fases principais, com diferentes objectivos associados, e que passarei a referir de seguida.

A primeira fase do trabalho teve como principal objectivo a determinação das vias de contaminação de queijo por *Listeria monocytogenes* em queijarias da Sub-Região Demarcada do Queijo de Castelo Branco. Para a concretização deste objectivo foi efectuada uma deslocação ao *Health Protection Agency* (HPA), em Londres, tendo sido aplicadas as técnicas moleculares RT-PCR, AFLP, PFGE e serotipagem molecular a um vasto conjunto de estirpes de *Listeria* spp., previamente seleccionadas a partir de isolados recolhidos ao longo de 10 anos a partir de amostras de queijo de ovelha, de leite cru, de solução de cardo, de salmoura, de solução fungicida, de fezes de ovelha e de zaragatoas a superfícies no interior das queijarias.

Os objectivos associados a esta deslocação foram:

- Caracterizar a diversidade genotípica de 185 estirpes pertencentes às espécies *Listeria monocytogenes, L. innocua e L. seeligeri;*
- Verificar a possível persistência de estirpes de *Listeria monocytogenes* nas queijarias e traçar as vias de contaminação;
- Estudar a possível associação entre a presença de *Listeria monocytogenes* isolada de leite cru excretado por uma ovelha com mamite subclínica, de queijo contaminado e da máquina de lavar os queijos.
- Implementar um novo ensaio *multiplex* PCR para a serotipagem de *Listeria monocytogenes,* previamente descrito (Doumith *et al*., 2004).

A segunda fase do trabalho teve por principal objectivo contribuir para o controlo do crescimento e sobrevivência de *Listeria monocytogenes* e outros no queijo de ovelha e também contribuir para o controlo do crescimento e sobrevivência de microrganismos indesejáveis. Partindo do conhecimento de que (1) a nisina é um agente conservante natural cuja aplicação se encontra autorizada em queijos curados, (2) a contaminação dos queijos está muitas vezes localizada apenas, ou em maior concentração, na casca, (3) o soro de leite é um sub-produto da indústria queijeira, altamente poluente, com boas propriedades funcionais, e que (4) o uso de revestimentos edíveis com propriedades antimicrobianas pode constituir uma forma de controlar eficazmente a presença deste patogéneo no queijo, desenvolveram-se e estudaram-se formulações de revestimentos elaborados com base numa matriz proteica de isolado proteico de soro de leite e com vários agentes antimicrobianos incorporados, entre eles a nisina.

Assim, foram definidos os seguintes objectivos:

- Caracterizar 219 estirpes de *Listeria monocytogenes* e *L. innocua*, incluindo os 185 isolados caracterizados por tipagem molecular, quanto à sua sensibilidade à nisina nas concentrações finais 5, 10, 50, 100 e 200 IU/ml, usando o método das placas de gradiente, em 3 condições ambientais: pH 5,5 e 20ºC, pH 5,5 e 37ºC, e pH 6,8 e 37ºC;
- Determinar a Concentração Mínima Inibitória (CMI) de nisina para as 219 estirpes, verificar a possível ocorrência de estirpes mais tolerantes e estimar a respectiva frequência;
- Avaliar a inibição do crescimento de uma estirpe seleccionada de *L. monocytogenes*, em meio líquido, em cultura estática e na presença de nisina na concentração de 50 IU/ml.
- Avaliar a virulência de um conjunto de 7 estirpes de *L. monocytogenes* por aplicação de um ensaio *in vitro* do tipo *Plaque-Forming Assay* (PFA) sobre uma monocamada de células animais HT-29;
- Estudar a substituição do revestimento comercial para queijos *Emulgest*, à base de acetato de polivinil, cuja segurança por ingestão não está comprovada, podendo constituir um perigo para a saúde dos apreciadores de queijo que consomem a casca;
- Desenvolver uma formulação para revestimentos de baixo pH (cerca de 3) para proteger o queijo, à base de isolado proteico de soro de leite, de glicerol, de ácidos

orgânicos, de nisina, e de natamicina e com propriedades edíveis e antimicrobianas contra um conjunto de microrganismos maioritariamente isolados de casca de queijo (*Listeria monocytogenes*, *Pseudomonas aeruginosa, Yarrowia lipolytica, Penicillium commune* e *P. chrysogenum)*;

- Estudar as características reológicas e mecânicas das soluções filmogénicas desenvolvidas e dos filmes obtidos por secagem, nomeadamente ao nível das propriedades de viscosidade, viscoelasticidade, % de elongação, tensão máxima de ruptura, tensão máxima de penetração e permeabilidade ao valor de água.

Este trabalho foi estruturado em sete capítulos e dois anexos, constituindo o capítulo I o capítulo introdutório no qual se faz uma breve revisão bibliográfica sobre os temas em estudo e onde se referem os objectivos do trabalho. Os capítulos II e III dizem respeito à determinação das vias de contaminação nas queijarias estudadas, para a qual foi necessária a aplicação de vários métodos de tipagem molecular. Relata-se ainda um caso de listeriose ovina associado a contaminação de queijo, ambiente e leite. O capítulo IV diz respeito à avaliação da sensibilidade dos isolados a diferentes concentrações de nisina, a dois valores de pH e de temperatura. Seguem-se os capítulos V e VI, os quais dão conta do desenvolvimento de revestimentos edíveis e com propriedades antimicrobianas, com o objectivo de inibirem microrganismos indesejáveis presentes na superfície do queijo. Por fim, no capítulo VII sumariam-se as principais conclusões deste trabalho e sugerem-se tópicos para trabalho futuro. De referir que cada um dos capítulos II, III, IV, V e VI constitui um artigo publicado ou submetido a publicação.

Dado que alguns dos isolados caracterizados no trabalho de mestrado foram usados nos estudos de tipagem molecular, de sensibilidade a nisina e de virulência, optou-se por incluir em anexo a tabela com a caracterização desses isolados (Anexo I), facilitando assim a sua consulta, se necessário. Em anexo encontra-se ainda a listagem de publicações e comunicações apresentadas no âmbito do presente trabalho de doutoramento (Anexo II).

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CAPÍTULO II

Association between a case study of asymptomatic ovine listerial mastitis and the contamination of soft cheese and cheese processing environment with *Listeria monocytogenes* in Portugal

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Association between a case study of asymptomatic ovine listerial mastitis and the contamination of soft cheese and cheese processing environment with *Listeria monocytogenes* in Portugal

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Running title: *Listeria monocytogenes* and ovine mastitis

Abstract

For 5 months, the udders of milking ewes, raw ewe's milk, cheese, and the plant and environment of a cheese manufacturer in Portugal were investigated using standard methods for the presence of *Listeria* spp. An association between subclinical mastitis and *Listeria monocytogenes* in a single lactating sheep was investigated by visual inspection of udders for signs of inflammation, application of somatic cell counts, the California mastitis test, pH measurement to milk, and culture of *L. monocytogenes* and *Staphylococcus* spp. To track the routes of contamination by *L. monocytogenes,* 103 isolates were characterized by molecular serotyping and amplified fragment length polymorphism, and a selection was further tested by pulsed-field gel electrophoresis. This study provides molecular and epidemiological evidence tracking the persistence of a single *L. monocytogenes* strain causing a subclinical udder infection without obvious inflammation in a single ewe. This infection was the likely source of contamination of raw milk that was subsequently used to produce unpasteurised milk cheese and resulted in a single strain of this bacterium colonizing the processing environment and the final cheese product.

Key words: *Listeria monocytogenes*; raw ewe's milk cheese; subclinical ovine mastitis; subtyping methods; microbial ecology

1. Introduction

Listeria monocytogenes is now recognized as a major foodbornepathogen that infects the unborn and newly delivered, pregnant women, the elderly, and those with underlying conditions (Farber and Peterkin, 1991). Consumption of ready-to-eat foods with extended shelf lives that are capable of supporting the growth of *L. monocytogenes* is most frequently associated with transmission (McLauchlin, 1996). These products favor disease transmission because this bacterium is widespread in the environment, survives the sanitation process and colonizes food manufacturing plants, and multiplies in various foods over a wide range of temperatures, including those used for refrigeration (Lundén *et al.*, 2003). Food with a high salt content was implicated with transmission of listeriosis, and this includes outbreaks as well as sporadic cases that have been linked to the consumption of soft and raw-milk cheese that have occurred in England (McLauchlin, 1996), France, (Goulet *et al.*, 1995), Italy (Gianfranceschi *et al.*, 2006), Sweden (Loncarevic *et al.*, 1995), Switzerland (Bille, 1990), the USA (Farber and Peterkin, 1991), North America, Europe, and Japan (Swaminathan and Gerner-Smidt, 2007).

Studies have been undertaken in the EU and the USA to trace transmission routes of this bacterium through dairy environments, and the presence of this pathogen in ewe's milk has been linked with fecal contamination during improper milking (Jacquet *et al.*, 1993; Loncarevic *et al.*, 1995; Pritchard *et al.*, 1995; Waak *et al.*, 2002; Pintado *et al.*, 2005). However, excretion of the organism into the ewe's milk as a result of intramammary infections has been less well studied (Tzora *et al.*, 1998). *L. monocytogenes* mastitis is uncommon in small ruminants; however, subclinical infections are rarely identified, and the overall disease burden is likely to be underestimated (Low and Donachie, 1997; Fthenakis *et al.*, 1998; Schöder *et al.*, 2003).

To identify and track *L. monocytogenes* through the food chain, typing methods for this bacterium are essential. Genotypic methods used to type *L. monocytogenes* include molecular serotyping (Doumith *et al.,* 2004), ribotyping (Saunders *et al*., 2003), amplified fragment length polymorphism analysis (AFLP) (Guerra *et al*., 2002) and pulsed-field gel electrophoresis (PFGE) (Graves and Swaminathan, 2001). These methods have been used alone or in various combinations to successfully compare *L. monocytogenes* isolates from food environments to better understand the ecology of this bacterium (Unnerstad *et al*., 1996; Guerra *et al*., 2002).

We describe elsewhere investigations into the contamination of raw ewe's milk from sheep farmers in Portugal that was the likely source for the presence of *L. monocytogenes* in cheese and cheese manufacturing environment (Pintado *et al.* submitted). The aim of this study was to further evaluate the possible relationship between *L. monocytogenes* from ewe's milk, cheese, and the cheese manufacturing environment with listerial mastitis.

2. Material and Methods

2.1. Cheese manufacturers

In early May 2004, the routine investigation for good hygiene and manufacturing practices revealed *L. monocytogenes* in a batch of cheese, ready to enter the cold rooms for the second stage of maturation, during which it is maintained at 12-14º C for a minimum of 40 days in an atmosphere of 70-80% relative humidity. Cheeses made in the plant were prepared from raw ewe's milk clotted by vegetable rennet made from the thistle-like cardoon flower. The cheeses were washed, brined, and matured in cold rooms as previously described (Pintado *et al*., 2005). *L. monocytogenes* contaminating this batch of cheese was also recovered from bulk milk from one particular producer. To track the origin of the cheese contamination, sheep's milk supplied to the plant from flocks of Awasi, Assaf, and Merino breeds (grazing in open fields with occasional silage feeding during bad weather) was tested for the presence of *L. monocytogenes* (Pintado *et al*., submitted for publication).

After the investigation, milk from this producer was received and processed separately in a pilot plant of the cooperative, and *L. monocytogenes* isolates from the contaminated milk were compared with those from the plant environment and from other products within this cooperative.

2.2. Microbiological analyses, examination, and sampling procedures

All samples were analysed for the presence of *Listeria* using standard methods by homogenization into buffered peptone water for 1 h at 20ºC, and then incubated in selective enrichment broths (Anonymous, 2004), followed by subculturing onto selective agars (Pintado *et al*., 2005; Leite *et al*., 2006). The levels of *Listeria* present were enumerated by direct subculture of dilutions of the homogenate onto selective agar (Oxford listeria selective agar; Merck, Darmstadt, Germany) as previously described (Pintado *et al*., 2005; Leite *et al*., 2006). Up to five isolated, characteristic colonies from each sample were subcultured from selective agar onto blood agar and chromogenic selective agar (ALOA; AES Laboratoire, Bruz, France) and identified using the API Listeria identification system (bioMérieux, Marcy l'Etoile, France) as previously described (Gameiro *et al*., 2007).

Samples of milk (10mL) and 5 or 25g samples of mature cheese rinds and cores together with cardoon macerate in tap water and brine solutions from this factory were sampled. Food and nonfood contact surfaces were also sampled using tissue swabs or Isolation Transwabs (LT; Medical Wire and Equipment, Corsham, England).

Milk samples were tested for the presence of staphylococci, and up to five isolated, characteristic colonies growing on selective agar were subcultured onto blood agar, inoculated into rabbit plasma broths (BD BBL™; Coagulase Plasmas, Dundalk, Ireland), and tested for the presence of coagulase using ISO method 1999:6888-1 (Anonymous, 1999). Presumptive *Staphylococcus* spp. were further identified using the API Staph identification system (bioMérieux). *Staphylococcus aureus* ATCC 25923 was included as a positive control in each batch of tests.

2.3. Examination of sheep and sampling procedures

Where *L. monocytogenes* was detected in milk, animals were further investigated for signs of subclinical intramammary gland inflammation by

determining the pH of the milk, estimating the somatic cell counts (SCC) using the Fossomatic method (Gonzalo *et al.,* 1993), California mastitis test (CMT; Bovi-Vet, Kruuse, Germany), and estimating levels of *Staphylococcus* spp. (Anonymous, 1999). Results from the CMT were scored as follows: 0, no gel formation (equivalent to an SSC of $\leq 6.5 \times 10^5$ cells/mL); 1, slight evidence of gel (8.7×10^5 cells/mL); and 2, clear viscous gel ($\geq 2.7 \times 10^6$ cells/mL). Udders of all individual ewes of the producer's flock were examined under veterinary supervision for visual signs of mammary gland inflammation. Any animals suspected to have mastitis was further investigated.

Samples of raw ewe's milk were taken both from the producer's bulk tank and from suspected animals for the presence of *Listeria*. For this investigation, milk from groups of up to 20 of the 400 ewes was first tested for SSC and/or the CMT, and if there was any evidence for mastitis (SSC value of $> 6.5 \times 10^5$ cells/mL or CMT values of 1 or 2), milk from individual animals was collected to test for the presence of *L. monocytogenes*. Samples of milk from an individual animal with mastitis (see below) were analyzed each day (morning and evening) and were colleted for 1 week between the 2nd and 8th of October for the presence of *Listeria.* Milk collected from the left and right halves of the udders was tested separately to establish if the whole gland was equally infected.

One ewe was found to have listerial mastitis (but without visible inflammation) and was quarantined from June to October 2004. Milk and fecal samples were also collected for examination during this quarantine period, and this ewe gave birth to a lamb in early September which died a couple of hours after birth. The corpse was frozen and sent to the Veterinary Investigation Centre for histopathological examination of the liver tissue and microbiological analyses of both the liver and the central nervous system.

Analysis of the variance of means and standard deviations, determination of mean significant differences of the bacterial counts, and SCC determinations were performed using SPSS version 14.0 (SPSS, Chicago, IL). p-Values of ≤ 0.05 with a 95% confidence interval were considered significant.

2.4. Confirmation and subtyping of *L. monocytogenes* isolates

DNA for real-time PCR, AFLP analyses, and molecular serotyping was prepared with either a MagNA Pure Compact system (Roche Diagnostics, Penzberg,

Germany) or a MagNA Pure LC system (Roche Diagnostics) using bacterial cells grown on horse blood agar in Columbia Blood Agar base (Oxoid, Hampshire, United Kingdom). A negative control of sterile distilled water was included as a no-template control with each batch of extractions. DNA samples were either used immediately or stored at -20ºC until required.

Further confirmation of identity as *L. monocytogenes* was preformed by real-time PCR with primers (MWG-Biotech AG, Ebersberg, Germany) and a hydrolysis probe (Oswell [Southampton, United Kingdom] and Eurogentec [Seraing, Belgium]) directed against a listeriolysin O gene fragment (*hlyA*) (Novga *et al.*, 2000). PCR products were detected using default settings of an Applied Biosystems 7700 Sequence Detector System (PE Biosystems, Foster City, CA). DNA extracted from *L. monocytogenes* NCTC 11994 and DNA extracted from *Listeria seeligeri* NCTC 4814 were used as positive and negative controls, respectively, for each batch of tests. Cycle threshold values between 15 and 37 were considered as positive for the amplification of the *hlyA* target sequence.

Molecular serotyping and AFLP analyses using *Eco*RI were performed on all *L. monocytogenes* isolates as described previously (Guerra *et al*., 2002; Doumith *et al*., 2004), and ethidium bromide-stained electrophoresis gels were recorded using the Gel Doc 2000 image capture system (BioRad Laboratoires, Hercules, CA). *L. monocytogenes* NCTC 11994 was included as a positive control and sterile distilled water as negative control in each batch of tests. Selected *L. monocytogenes* isolates that were indistinguishable by both serotyping and AFLP analysis were further tested by PFGE as described previously (Graves and Swaminathan, 2001) with minor modifications: the isolates were subcultured onto Columbia Blood Agar (Oxoid); cells were removed to plastic tubes containing 1 mL of TE buffer; the plugs were sliced in 4-5mm portions. For each batch of gels one wild-type *L. monocytogenes* (H2446, CLIP 77873 Institut Pasteur, Paris, France) and *Salmonella enterica* serovar Braenderup (H9812) were included as controls. Pattern analyses were performed using BioNumerics software versions 3 and 4 (Applied Maths, Sint-Martens-Latem, Belgium) using unweighted pair group method with arithmetic means analyses with Dice coefficient.

3. Results

During routine investigation of a cheese manufacturer in early May 2004, *L. monocytogenes* was detected in cheese and in the production environment of a single manufacturer. To track the origin of the cheese contamination, sheep's milk in the bulk tank supplied to the plant was analysed, and *L. monocytogenes* was isolated. On examination of all the lactating animals during the first week of this study, no evidence of inflammatory symptoms was observed by visual inspection of their udders and results of triplicate SCC determinations performed on the entire bulk milk during mid to end of lactation (in May 2004) ranged from $9x10^5$ to $1.1x10^6$ cells/mL. After sampling milk from groups of 20 ewes and excluding those with a positive CMT (score ≥1), the counts reduced to between 0.9 and $1x10^5$ cells/mL (CMT score 0). The SCCs increased to 1.1 to $1.6x10^6$ cells/ml (CMT score >1) in summer during July to August but decrease to $8.7x10^5$ cells/mL (CMT score 1) in the beginning of autumn by mid September. A single Merino ewe was subsequently found to have listerial mastitis, and quarantined milk samples collected from the left (infected) or right (uninfected) udders halves gave mean SSC counts of $2.7x10^6$ cells/mL (CMT scores 2) and $6.5x10^5$ cells/mL (CMT score 0). Milk yields were reduced in the left half udder of this animal by between 20% and 60 % of the average daily volume.

Histopathological analyses of the lamb born in early September to the ewe with mastitis showed several foci of cell necrosis in the liver tissue, which indicated an intra-uterine infection produced before delivery. *Staphylococcus lentus* and *Corynebacterium* group F were both recovered from brain tissue, and *Staphylococcus equorum* alone was isolated from the liver. No *Listeria* spp. were detected by microbiological culture or by histopathology in any tissue samples.

A total of 53 samples of cheese (36) and environmental swabs and feces (17) were tested for the presence and levels of *Listeria* between May and July 2004 (Table 1). Included among the contaminated products were the ewe's milk, three independently produced and matured batches of cheese, and environment sites at the pilot plant in the cooperative (Table 1). All three batches of cheese (collected on the 45th, 49th, and 53rd day of the 55th ripening period) showed significantly higher mean levels of *L. monocytogenes* in the rind than in core of the cheese ($p \leq 0.05$). *L. monocytogenes* and *Listeria innocua* were recovered from swabs taken from brushes

from one machine located in the cheese washing area (Table 1). Despite extensive sampling, this was the only factory site where *Listeria* spp. were detected.

Table 1. Detection and enumeration of *Listeria* spp. in cheese, the production environment, and sheep feces

Description		Detection	Enumeration (mean CFU/g)
		Cheese:	
45 d Maturation	Core	*L. monocytogenes*	$1.0x10^2$
	Rind	*L. monocytogenes*	$2.1x10^5$
49 d Maturation	Core	*L. monocytogenes*	$< 1.0x10^2$
	Rind	*L. monocytogenes*	$1.0x10^5$
53 d Maturation	Core	*L. monocytogenes*	$1.4x10^3$
	Rind	*L. monocytogenes*	$4.5x10^4$
		Environmental:	
Swabs of cheese	Brush	*Listeria innocua*	ND
washing machine	Handle	*L. monocytogenes*	ND
	Handle	*L. innocua*	ND
Ewe´s feces		*L. innocua*	ND

Mean differences are significant for $p \leq 0.05$; ND, not determined; d, days; CFU, colony forming units.

Bacteriological results of 28 milk samples collected in both the morning and the evening between the week of 2nd and 8th of October from the two halves of the udder of the affected ewe are shown in Table 2. There was significant variation ($p \leq 0.05$) in the mean levels of *L. monocytogenes* and *Staphylococcus* spp. between the right and left sides of the udder, and this variation was consistent for the milk collected during the whole week. The pH of the milk collected between 6th and 8th of October ranged from 6.58 to 6.72 on the right side and 6.78 to 6.91 on the left (contaminated) side, and was significantly different. All staphylococci were coagulase test negative and a selection of 22 representative isolates were all confirmed as *Staphylococcus* spp. by colonial morphology, Gram stain, and catalase

reaction. Isolates were further identified as *Staphylococcus xylosus* (3 isolates), *S. lentus* (1 isolate) and *S. cohnii* (1 isolate), and the remaining 17 could not be identified to species level. *L. innocua* alone was isolated from the feces of the ewe with listerial mastitis (Table 1).

Table 2. Results of detection of *Listeria monocytogenes* and *Staphylococcus* spp. in milk from a single ewe with mastitis

Day	Time	*L. monocytogenes* (CFU/mL)		*Staphylococcus* spp. (CFU/mL)	
		Half of udder[a]			
		Right	Left	Right	Left
2 October	Morning	<10	$3x10^1$	<10	$1.7x10^3$
	Evening	<10	$6x10^1$	<10	$7x10^1$
3 October	Morning	<10	$1.2x10^3$	<10	$7.9x10^2$
	Evening	<10	$1.4x10^3$	$1x10^1$	$1x10^2$
4 October	Morning	<10	$5x10^2$	<10	$5.9x10^2$
	Evening	<10	$9x10^1$	$7x10^1$	$9x10^1$
5 October	Morning	<10	$3.5x10^1$	$2x10^1$	$6x10^1$
	Evening	<10	$3.5x10^1$	$2x10^1$	$2.3x10^3$
6 October	Morning	<10	$1x10^2$	$1.3x10^2$	$3.2x10^3$
	Evening	<10	$3x10^1$	<10	$2.7x10^3$
7 October	Morning	<10	$4.3x10^1$	$2x10^1$	$4.6x10^3$
	Evening	<10	$2x10^1$	<10	$7 x10^2$
8 October	Morning	<10	$4x10^1$	$3x10^1$	$5.1x10^2$
	Evening	<10	$1x10^2$	$8x10^1$	$2.5x10^2$

Mean CFU are significant for $p \leq 0.05$; ND, not determined; CFU, colony forming units;
[a] Differences of right and left.

A total of 119 *Listeria* isolates (103 *L. monocytogenes* and 16 *L. innocua*) were obtained from raw ewe's milk, sheep feces, cheese, and the manufacturing environment from the factory. There were 37 *L. monocytogenes* isolates from cheese, 56 from milk, and 10 from environmental swabs taken within the factory. Fifteen *L. innocua* were isolated from swabs taken within the factory and one isolate (described above) was collected from the sheep's stools. The origins of these isolates are described in Table 1. Forty eight isolates from milk (14), cheese (30) and the factory

environment (4), previously identified by conventional methods as *L. monocytogenes,* were all re-confirmed by real-time PCR. Molecular typing on all 48 *L. monocytogenes* isolates showed that all belonged to serovar 4b, AFLP type IV-1 (Table 3). Six isolates from milk, cheese (rind and core) and the factory environment were further analyzed by *Asc*I digestion followed by PFGE and all were indistinguishable and classified as type 11: five of these isolates were further analysed by PFGE after an *ApaI* digestion, and all produced the same profile and were indistinguishable.

Table 3. Molecular characterization of selected *Listeria* spp. isolates

Isolates		Subtype		
Source (number of isolates)	Conventional species identification	PCR serogroup	AFLP type	PFGE type *Asc*I , *Apa*I
Cheese 45d, rind (5) [a]	*Listeria monocytogenes*	4b	IV-1	ND
Cheese 45 d, rind (1)	*L. monocytogenes*	4b	IV-1	11
Cheese 45 d, core (4)	*L. monocytogenes*	4b	IV-1	ND
Cheese 49 d, rind (6)	*L. monocytogenes*	4b	IV-1	ND
Cheese 49 d, core (3)	*L. monocytogenes*	4b	IV-1	ND
Cheese 49 d, core (1)	*L. monocytogenes*	4b	IV-1	11, 1
Cheese 53 d, rind (6)	*L. monocytogenes*	4b	IV-1	ND
Cheese 53 d, core (3)	*L. monocytogenes*	4b	IV-1	ND
Cheese 53 d, core (1)	*L. monocytogenes*	4b	IV-1	11, 1
Washing brush central sticks (1)	*Listeria innocua*	ND	XX	ND
Washing brush left sticks (3)	*L. monocytogenes*	4b	IV-1	ND
Washing brush left sticks (1)	*L. monocytogenes*	4b	IV-1	11, 1
Washing brush right sticks (2)	*L. innocua*	ND	XX	ND
Milk left half of udder (12)	*L. monocytogenes*	4b	IV-1	ND
Milk left half of udder (2)	*L. monocytogenes*	4b	IV-1	11 , 1
Ewe's fecal sample (1)	*L. innocua*	ND	XXII+2	ND

[a] Number of isolates are shown in parentheses; ND, not determined; d, days.

Of the fourteen isolates from ewe's milk collected between 2nd and 8th of October from the left half side of the mammary gland, all were serovar 4b, AFLP IV-1. Two of these isolates (collected on the 2nd and 8th of October) had the same PFGE profiles like the isolates from the cheese and factory environment. The 18 isolates obtained from ewe's milk from other milk suppliers all showed different types to that

from the affected ewe and are fully described elsewhere (Pintado *et al*., submitted for publication).

Four *L. innocua* isolates from swabbing factory machinery and from ovine feces were characterized by AFLP. All four isolates generated a distinct profile designated as XX, as previously observed on environment isolates in this factory (Matos, 2003).

4. Discussion

The ecology and transmission of *L. monocytogenes* in ruminants is poorly understood (Nightingale *et al*., 2004), and there has only been a single report of a direct association between cases of listerial mastitis in sheep and contamination of raw cheese and the cheese production environment (Schöder *et al*., 2003); no molecular characterization of isolates was performed in this study. Further, farm-scale raw ewe's cheese production is common in Portugal (Pintado *et al*., 2005; Leite *et al*., 2006) and is a business of growing economic importance, especially because these products are typical of South European food.

In the study described here we provide molecular epidemiological evidence tracking the persistence of a single *L. monocytogenes* strain causing subclinical udder infection without obvious inflammatory signs in a single ewe. Further, this infection was the likely source of contamination of raw milk used to produce an unpasteurized milk cheese and resulted in this bacterium colonizing the processing environment and the final cheese. Subclinical infections of this kind have been reported with minimal visible signs and with SSC levels of > 1 to 2.96×10^6 and $<5 \times 10^5$ to 1.0×10^6 cells/mL in milk from infected and uninfected animals, respectively (Fthenakis *et al*., 1998; Contreras *et al*., 2007). These SSC levels are in contrast to the $<10^6$ cells/ mL observed with acute intramammary gland inflammation due to coagulase negative staphylococci (Ariznabarreta *et al*., 2002). Characterization of *L. monocytogenes* from ovine diseases has rarely been described, although serotyping data on isolates from 50 cases of abortion, encephalitis, and septicemia in Scotland were reported in the early 1990s (Low *et al*., 1993). In this study, only 18% of *L. monocytogenes* isolates were serovar 4b, which is the predominant serovar in both sporadic cases and

outbreaks of human disease (Farber and Peterkin, 1991; Low *et al*., 1993; McLauchlin, 1996; Swaminathan and Gerner-Smidt, 2007). Longitudinal data on the distribution of *L. monocytogenes* serovars in Portuguese ewe's milk cheese have shown serovar 4b to be consistently more commonly detected than other serotypes in the two main regions of farm scale production (Pintado *et al*., 2005) and as well in production areas apart from these (Chambel *et al*., 2007); however, no epidemiological linkage to human infections has to date been detected (Leite *et al*., 2006). The ovine mastitis reported here was identified as subclinical and linked to a strain of *L. monocytogenes* serovar 4b that was subsequently shown to be virulent in an *in vitro* HT29 human colon adenocarcinoma tumour cells invasion assay (Pintado *et al.*, submitted). This strain showed to be as virulent as the EGDe virulent reference strain reported in the literature and used in the assay. The cause of death of the lamb from the affected ewe was probably not associated with listeriosis, and this ewe itself subsequently died and could not be further investigated.

This report illustrates the need to investigate subclinical mastitis in dairy ewes and the importance of rapid detection to exclude *L. monocytogenes* from the food chain. It is essential for the safety of raw ewe's milk cheese to implement monitoring of all animals by CMT (is easy to do at the parlor), because the diagnosis of this illness is difficult with minimal inflammatory symptoms after onset of mammary gland infection (Tzora *et al*., 1998). In the incident reported here, the milk producer did not recognize that a ewe was infected despite the considerable levels of *L. monocytogenes* and staphylococci excreted into the milk. Further, the single infected ewe occurred in a flock of 400 animals resulting in the milk from this animal being diluted about 700-fold in the bulk tank, and contamination with *L. monocytogenes* was only detected postproduction in a batch of cheese. This led to the complete destruction of the entire batch of cheese from this milk producer and the revoking of a licence for milk supply to this co-operative's plant (Pintado *et al*., submitted). *L. monocytogenes* in cheese made in the pilot plant with this contaminated milk did not significantly decrease in either the core or the rind during the 53 days of ripening, although there were considerably higher levels of the bacterium in the rind. The persistent contamination of food contact surfaces (i.e., brushes used for the washing step) may be the reason for these higher levels in the rind together with subsequent multiplication of the bacterium within the outer portions of this food matrix. As was

previously reported for studies conducted in other regions of raw ewe's milk cheese production in Portugal, extended ripening periods do not prevent *L. monocytogenes* contamination in the final product (Gameiro *et al*., 2007). However, control of this bacterium lies in maintaining good udder health together with use of good hygienic and manufacturing practices in the food production environment and in avoiding postprocess contamination. The inability to detected cases of ovine mastitis due to *L. monocytogenes* was also recognized as important with ewe's milk cheese production in Austria (Schöder *et al*., 2003) which further supports the need for routine CMT and SSC (as previous comment) screening of lactating ewes. Raw ewe' milk cheese is a farm-scale business where intra-mammary infections can decrease the yield of milk by more than 50% (Leitner *et al*, 2008) thus causing considerable economical losses and increased food safety risks.

Further studies are needed to understand the relationship between pathogens and mammary gland immune response in milking ewes. However, this study demonstrates the potential usefulness of SCC and CMT as tools for the diagnosis of subclinical mastitis and to identify individual ewes within a flock that may be the source for contamination within cheese manufacturing plants.

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CAPÍTULO III

Routes of contamination by *Listeria monocytogenes* in ewe’s milk cheese processing plants in Portugal

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Routes of contamination by *Listeria monocytogenes* in ewe's milk cheese processing plants in Portugal

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Running title: *Listeria monocytogenes* in a ewes milk cheese plant

Abstract

Aims: Between 1995 and 2004, raw ewe's milk, cheese, and the plant and environment of cheese manufacturers in three different regions in Portugal were investigated to track the routes of contamination by *Listeria monocytogenes*.

Methods and Results: A total of 473 samples were analyzed using standard methods for the presence of *Listeria* spp. and 185 isolates were recovered. *L. monocytogenes* was isolated from cheese (81), milk (32) and environment (10): one hundred isolates were from region A, 20 from B and 3 from C. One hundred and twenty three isolates were characterized by molecular serotyping and amplified fragment length polymorphism, and a selection of these were further tested by pulsed-field gel electrophoresis. Cluster analysis of isolates using the different subtyping methods identified similar groupings. Some representative isolates were also assayed for virulence potential on HT 29 cells and tested positive.

Conclusions: Contaminated ewe's milk from different producers was the likely source of contamination of cheese. In addition to the contamination of milk as a

hazard in this process, the washing areas and brine solution were also hazardous for hygienic cheese production.

Significance and Impact of Study: As a result of this investigation, hazardous points are identified in the process and suggestions for control were given that resulted in eradication of environment contamination and improvements in safety production.

Key words: *Listeria monocytogenes*; raw ewe’s milk cheese; subtyping methods; microbial ecology.

1. Introduction

Listeria monocytogenes is a bacterium which, following a series of outbreaks in Europe and North America in the 1980s, is now recognised as one of the major food-borne pathogens (Farber and Peterkin, 1991). Infection occurs in the unborn and newly delivered infants, pregnant women, the elderly and in those with pre-existing severe underlying illness (Swaminathan and Gerner-Smidt, 2007). The properties of *L. monocytogenes* favour food-borne transmission: the bacterium is widespread in the environment, survives the sanitisation process in food manufacturing plants, and can multiply under certain foods at conditions of high salt and over a wide range of temperatures, including those used for refrigeration (Lundén *et al*., 2003). Raw ewe’s milk cheese most traditionally consumed in Portugal are made by addition of salt and use cold ripening to mature until a final semi-soft creamy texture (Pintado *et al*., 2005). Sporadic cases and outbreaks of listeriosis are most usually associated with consumption of ready-to-eat foods which have extended (usually refrigerated) shelf lives and are capable of supporting the growth of *L.monocytogenes*, e.g. pâté, smoked fish, salads, sandwiches and dairy products including soft cheese (McLauchlin, 1996). Consumption of contaminated soft cheeses had been particularly associated with transmission of listeriosis and cases implicated with this food have been identified in England, France, Italy, Sweden, Switzerland, and the USA (Bille, 1990, Goulet *et al.,* 1995, Loncarevic *et al.,* 1995, McLauchlin 1996, Gianfranceschi *et al*., 2006).

Since this bacterium is common in the environment, ingestion of *L. monocytogenes* via consumption of contaminated foods is probably frequent.

However the incidence of infection is low: rates of <1 to >7 cases per million of the population per year have been reported for European countries (de Valk *et al*., 2003). Surveillance data on listeriosis in Portugal is probably insensitive (Almeida *et al*., 2006), and reporting to the Portuguese Health Authority is not mandatory. However, the Pathology Laboratory of the University Hospital of Coimbra recorded 15 human cases of listeriosis between 1997 and 2003 (Leite *et al*., 2006) and, since this laboratory serves a population of 500,000, represents a minimum average annual incidence of 5 cases per million, which is a similar incidence to other European countries.

To trace transmission routes of *L.monocytogenes,* reliable typing methods to successfully track this bacterium through the food chain are essential (Unnerstad *et al*., 1996; Sauders *et al*., 2003). Genotypic methods are now widely used and these include amplified fragment length polymorphism analysis (AFLP; Guerra *et al*., 2002) and pulsed-field gel electrophoresis (PFGE; Graves and Swaminathan, 2001).

A high incidence of *L.monocytogenes* contamination of ewe's milk cheese in Portugal was previously reported (Pintado *et al*., 2005; Chambel *et al*., 2007). The aims of this study were to: analyse the distribution of *Listeria* species and *L. monocytogenes* strains isolated from raw ewe's milk, cheese and cheese manufacturing environments; examine the possible persistence of *L. monocytogenes* strains in these environments; track *L. monocytogenes* through raw ewe's milk cheese processing plants to set control points; and contribute with recommendations to the improvement of procedures used in cheese manufacture.

2. Material and methods

2.1. Cheese manufacturers

Samples were collected between 1995 and 2004 from milk, cheese and the cheese processing environment of a series of semi-soft cheese producers designated A-N (Pintado *et al*., 2005). Producers used manual or mechanical milking to obtain ewe's milk from flocks ranging from less than 80 to around 400 sheep. The cheese was either made twice daily or the evening and morning milking were mixed and kept under cooling conditions until processed. Cheese plant A is a co-operative, in region

A that receives milk from the herds of associated members (P1, P2, P3, P4, P6, P7, amongst others) and produces and sells matured cheeses. Cheese plants B, C, D, E, F, G, H, I, J, L, M are small cheese-manufacturers, located in region B (30-40 Km away from region A), and that sometimes used the refrigerated room of the co-operative in region B to mature their cheeses. Cheese plant N is a small cheese-manufacturer located in region C (80-90 Km from region A and 50-60 Km from region B). Cheese from regions A and B are of the Castelo Branco variety which is made from raw ewe's milk clotted by vegetable proteases prepared by macerating cardoon flowers (*Cynara cardunculus*) in tap water. The curds are cut and pressed to drain the whey, shaped into flat cylinders and either brined (plant A) or surface rubbed with coarse salt (plants B, C, D, E, F, G, H, I, J, L, M) and matured in cool rooms (Pintado *et al*., 2005). During their 40 d minimum maturation period, the cheeses are washed at regular intervals and a characteristic slimy smear appears on their surface. Cheese from region C (plant N) is of the Tolosa variety and is also produced in a traditional manufacture way involving the use of a surface treatment with coarse salt.

2. 2. Sampling procedures

Samples of raw ewe's milk were collected from both the producers' and manufacturers' bulk tanks together with whole cheese slices, rind and core from mature cheeses were analysed for the presence of *Listeria* as previously described (Pintado *et al*., 2005).

Direct swabbing using swabs of *Listeria* Isolation Transwab (LIT, Medical Wire and Equipment, Corsham, England) was used within food manufacturing areas to analyse both surfaces in contact with curd or cheese (e.g. wire knives, vats, moulds, hands, cheese washing devices, shelves, templates of working surfaces, towels and clothes) and non-food contact surfaces (drains, PVC ribbons and floors). Samples of fungicide solutions, cardoon macerate, as well as brine solutions used in this cheese production were also sampled for the presence of *Listeria*.

2.3. Detection and subtyping of *Listeria* isolates

Samples were tested for the presence of *Listeria* using standard methods (IDF 143:1995 or ISO 11290-1) as previously described (Pintado *et al*., 2005; Leite *et al*., 2006) by selectively enriched culture, subculturing onto selective agars (Oxford or

Palcam listeria selective agars, Merck, Darmstadt, Germany) and by direct subculture onto chromogenic selective agars (ALOA, AES Laboratoire, Bruz, France). Isolated colonies growing on selective agars that were characteristic for *Listeria* spp. were subcultured onto blood agar and identified using the API Listeria identification system (BioMerieux, Marcy l'Etoile, France) as previously described (Pintado *et al.*, 2005; Leite *et al*., 2006).

DNA for AFLP analysis and molecular serotyping was extracted using a MagNA Pure Compact system (Roche Diagnostics, Germany) or a MagNA Pure LC system (Roche Diagnostics) using 10µl loopful of pure bacterial growth on 5% (v:v) horse blood agar in Columbia Blood Agar base (Oxoid, Hampshire, UK). A negative control of sterile distilled water was included as a non-template control with each batch of extractions and DNA samples were either used immediately or stored at -20ºC until required.

Molecular serotyping and AFLP analysis with *Eco*RI were performed as described previously (Doumith *et al*., 2004; Guerra *et al*., 2002) and ethidium bromide stained electrophoresis gels were recorded using the Gel Doc 2000 image capture system (BioRad Laboratories).

Selected *L. monocytogenes* cultures were analysed by PFGE and included isolates indistinguishable by serotyping and AFLP analysis, with a common origin. PFGE was performed as described previously (Graves and Swaminathan, 2001) with minor modifications. For the PFGE analysis, four standard strains were included as controls for each batch of PFGE gels: two wild type *L.monocytogenes* clinical isolates from the UK (H5054616 and H5098575), one wild type *L.monocytogenes* from the Institut Pasteur (Paris, H2446, CLIP 77873), and *Salmonella enterica* serovar Braenderup (H9812).

AFLP band patterns were examined visually and roman numerals were assigned to individual patterns with more than two band differences. When an individual pattern showed differences of one or two bands, these were designated with +1 or 2 /-1 or 2. PFGE patterns were compared with one another by visual analysis and designated by a number. Pattern analysis was also performed using BioNumerics software version 3 and 4 (Applied Maths, Belgium) using unweighted pair group method with arithmetic means analysis (UPGMA) with the Dice coefficient.

2. 4. Virulence evaluation of *Listeria monocytogenes* isolates

Seven representative isolates from cheese (n=1), raw milk (n=5), and environment (n=1) were selected for characterization using an *in vitro* virulence assay. Virulent EGDe strain (serovar 1/2a) was used for each experiment as a reference strain. All strains were maintained at –80ºC in Tryptic Soy Broth (Biokar Diagnostics) with 15% (v/v) of glycerol and subcultured twice at 37ºC along 18 to 24 hours before use onto TSYEA medium.

Virulence potential was studied using an *in vitro* cell test based on a plaque-forming assay (PFA) with human adenocarcinoma cell line (HT-29) monolayer, set routinely in this laboratory as described by Neves *et al.* (2008) and based on virulence assessment explained by *Roche et al.* (2001). The pathogenic potential of isolates was expressed as the mean log of the number of plaques formed for 10^7 *Listeria* deposited per well in 96 wells plates (Nunc, Roskilde, Denmark). Mean values were calculated from at least two independent experiments performed in duplicate.

3. Results

3.1. Occurrence of *Listeria* spp. and subtyping of *Listeria* isolates

A total of 473 samples from sheep milk, cheese and manufacturing plants were tested for the presence of *Listeria,* from which 185 isolates were recovered (Table 1): 123 were *L. monocytogenes*, 62 *L. innocua* and 2 *L. seeligeri.* The average rate of contamination by *L. monocytogenes* was 28, 6% (36:126) for cheese (whole slices), 6, 3% (11:176) for milk and 5, 8% (10:171) for environment. Contamination with several *Listeria* spp. was simultaneous in the same sample.

All isolates were tested by serotyping and AFLP analysis. Following analysis of serotyping and AFLP typing results, together with considerations on their origins of isolation, 31 isolates of *L. monocytogenes* from raw milk and cheese, and 5 from the environment were analysed by PFGE.

Table 1. Distribution and origins of *Listeria* spp. associated with ewe's milk cheese in Portugal

Year of Isolation	Region (code)	Sample type	Isolation site	Isolates of *L.monocytogenes* (other *Listeria* species)
1995	A	Castelo Branco Cheese	Cheese plant A(1)	28 (0)
1995	B	Castelo Branco Cheese	Cheese plants C, D, F, G, I, J, L, M(2,4)	20 (4)
1998	C	Tolosa Cheese	Cheese plant N(3)	3 (6)
2000	A	Castelo Branco Cheese	Cheese plant A	0 (11)
2000	A	Raw ewe´s milk	Milk producers (P1, P3, P4, P5, P6, P7) and bulk tank milk in cheese plant A	18 (4)
2000	A	Cheese brine	Cheese plant A	1 (9)
2000	A	Environment	Cheese plant A	2 (20)
2000	A	Cardoon thistle extract	Cheese plant A	1 (3)
2000	A	Fungicide solution	Cheese plant A	2 (1)
2004	A	Castelo Branco Cheese	Cheese plant A	30 (0)
2004	A	Raw milk from one ewe	Milk producer P2	14 (0)
2004	A	Environment	Cheese plant A	4 (3)
2004	A	Animal faeces	Farm P2	0 (1)

(1) Cheese plant A is a co-operative, in the region A, that received milk from the herds of associated members (P1, P2, P3, P4, P5, P6, P7, amongst others) and made, matured and sold the cheeses.

(2) Cheese plants C, D, F, G, I, J, L, M are small cheese-houses, located in region B (30-40Km away from region A), and sometimes used the refrigerated room of the co-operative, located in B region to mature their cheeses.

(3) Cheese plant N is a small cheese-house, located in region C (80-90Km away from region A and 50-60Km away from region B).

(4) Cheese plants B, E, and H tested *Listeria* negative.

Identical AFLP results were obtained using DNA extracted from 28 *L.monocytogenes* and 10 *L. innocua* isolates which were independently tested on more than one occasion (Figure 1).

Of the 36 selected *L. monocytogenes* cultures which were all characterised by serotyping, AFLP and PFGE (Table 2), some clustering was recognised by all three methods. For example, of the ten cultures which were serovar 4b and AFLP type IV-1, eight of these were PFGE type 11 (Figure 2). However, *L. monocytogenes* cultures of the same serovar and AFLP type did not always generate the same PFGE profiles. For example, there were 6 distinct PFGE profiles recognised amongst the 9 cultures which were serovar 4b, AFLP type V.

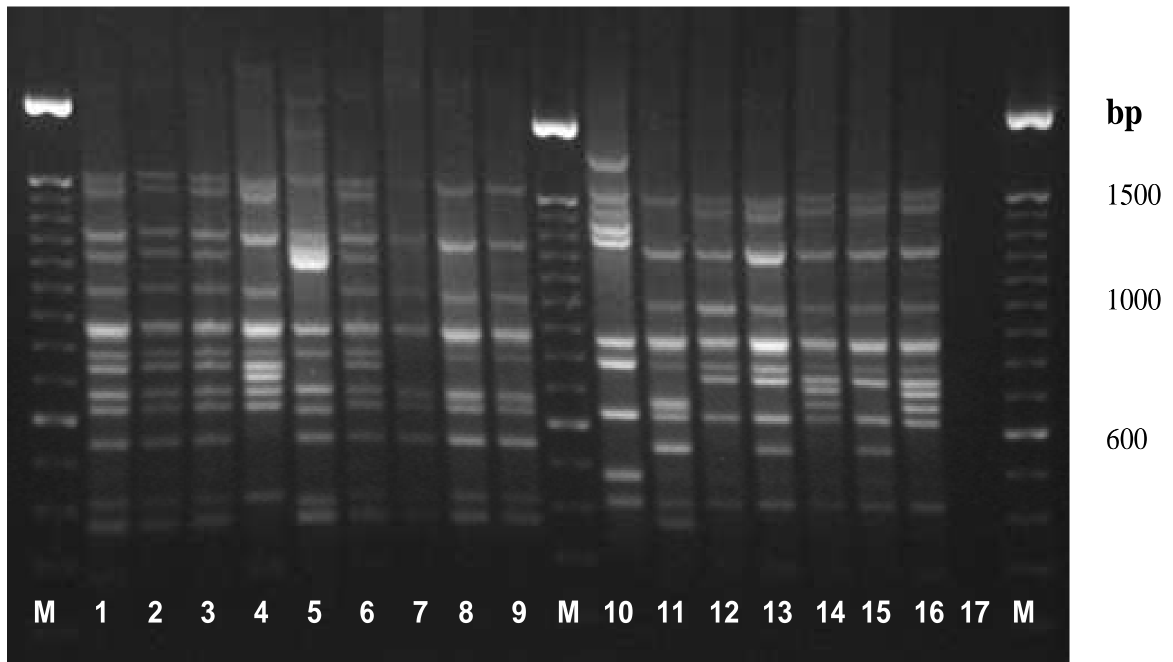

Figure1. Representative band patterns of AFLP types.
L. monocytogenes (1,2,3,4,6,7,8,9,11,12,13,14,15,16)*, L. innocua* (5) and *L. seeligeri* (10) . Lane M is the Marker. Lanes 1, 2, 3, 6 – AFLP type II; Lanes 4, 14, 16 - AFLP type I; Lane 5 – AFLP type XX; Lanes 7, 8, 9, 11 – AFLP type V; Lane 10 – AFLP type XXX-1; Lane 12 – AFLP type XV; Lane 13, 15 – AFLP type IV-1; Lane 17 – Negative control.

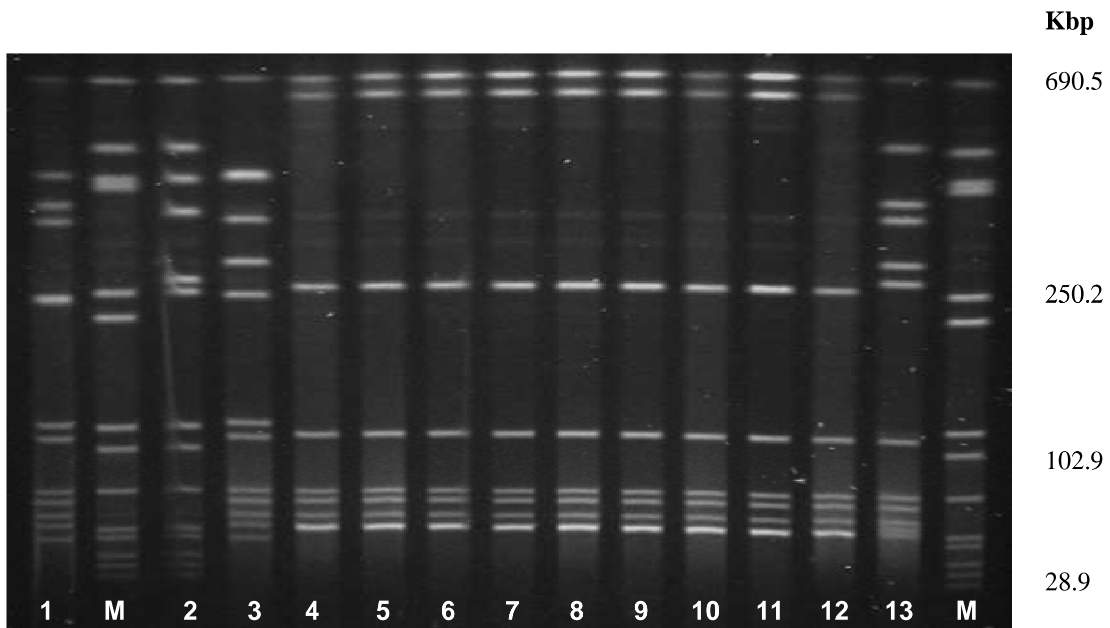

Figure2. PFGE separation of *AscI* macrorestriction fragments of *Listeria monocytogenes* genomic DNA.
Lane 1 – PFGE type 8; Lane 2 – PFGE type 9; Lane 3 – PFGE type 10 ; Lanes 4, 5, 6, 7, 8, 9, 10, 11, 12 – PFGE type 11; Lane 13 – PFGE type 18. Lanes M contain standard / reference CLIP 77873.

Amongst the 123 *L. monocytogenes* isolates that were tested by both serotyping and AFLP analysis, four of the six AFLP patterns were serotype specific: AFLP types I, V and XV for serovar 4b, and XIV for 1/2a. AFLP pattern II and IV-1 were common to strains of serovars 1/2b and 4b. AFLP profiles XX, and XXII were specific for the *L. innocua* isolates. The two isolates of *L. seeligeri* both generated the same AFLP types and indistinguishable patterns.

3.2. Routes of transmission

The results shown in Table 2 demonstrate considerable heterogeneity amongst the isolates tested. For example, in 1995, 2000 and 2004, 8 distinct *L.monocytogenes* strains were recovered from cheese, factory sites and equipment in plant A, together with 5 distinct strains in 2000 recovered from raw bulk milk, two of which were

recovered from the plant, see further discussion below. Furthermore, 13 of the 18 isolates from cheese made at plant A in 1995 selected for characterization by all typing methods differed from isolates from milk obtained from those producer members in the year of 2000 or with any other cheese samples except those outlined below. However, two of the isolates from cheese in 1995 were the same serotype, AFLP and PFGE types as an isolate from a towel used to clean the cheese after the washing step in 2000 (serovar 1/2b, AFLP XV and PFGE 6; Table 2). The cardoon extract used by plant A to curd the milk was found to be contaminated with *L. monocytogenes* of the same type (serovar 4b, AFLP V and PFGE 15; Table 2) as an isolate from one of the suppliers of ewe's milk (producer P4) to this plant. The cheese washing machine in plant A used to wash cheese during the maturation step of this kind of cheese, was also contaminated by the same type of *L. monocytogenes* as was recovered from producers P6 and P7 (serovar 1/2b, AFLP II and PFGE 17; Tabl 2).

Two isolates of *L. monocytogenes* collected a week apart from milk of producer P2 in 2004, were indistinguishable from isolates recovered from cheese and a cheese washing machine from producer A, as well as from cheese from a different producer and region in 1995 (serovar 4b, AFLP IV-1 and PFGE 11; Table 2). This similarity was further confirmed by obtaining the same PFGE profile using a further *Apa*I digestion amongst seven of the isolates (Table 2). This led to the investigations of flocks from producers which supplied milk, to determine the origin of the contamination of plant A. Investigation of the factory practices showed that for example, the cardoon extract contamination was attributed to inefficient cleaning and sanitation of a bucket that contained milk residues. Following the results of these investigations it was concluded that the likely original source of contamination was from the milk of producer P2.

Table 2. Characterization and origin of selected *L. monocytogenes* isolates

Year	Source	Producer / Region	Serovar or serovar group	AFLP type	PFGE type Asc*I* (Apa*I*)
1995	Castelo Branco Cheese	A / A	1/2b	XV	8
1995	Castelo Branco Cheese	A / A	1/2b	XV	6
1995	Castelo Branco Cheese	A / A	1/2b	XV	6
1995	Castelo Branco Cheese	A / A	4b	V	2
1995	Castelo Branco Cheese	A / A	4b	V	5
1995	Castelo Branco Cheese	A / A	4b	V	2
1995	Castelo Branco Cheese	A / A	4b	V	3
1995	Castelo Branco Cheese	A / A	4b	V	3
1995	Castelo Branco Cheese	C / B	4b	I	1
1995	Castelo Branco Cheese	C / B	4b	IV-1	11 (1)
1995	Castelo Branco Cheese	C/ B	4b	IV-1	11 (1)
1995	Castelo Branco Cheese	D / B	1/2b	XV	7
1995	Castelo Branco Cheese	D / B	1/2a	XIV	9
1995	Castelo Branco Cheese	F / B	4b	V	4
1995	Castelo Branco Cheese	G / B	1/2b	IV-1	10
1995	Castelo Branco Cheese	I / B	4b	I	1
1995	Castelo Branco Cheese	J / B	4b	I	1
1995	Castelo Branco Cheese	L/B	4b	I	1
1998	Tolosa Cheese	N / C	4b	IV-1+1	12
1998	Tolosa Cheese	N / C	4b	II-1+1	13
2000	Raw ewe´s milk from churns	P1 / A	4b	I	14
2000	Raw ewe´s milk from churns	P4 / A	4b	I	14
2000	Raw ewe´s milk from churns	P4 / A	4b	V	15
2000	Raw ewe´s milk from churns	P5 / A	4b	V	16
2000	Raw ewe´s milk from churns	P6 / A	1/2b	II	17
2000	Raw ewe´s milk from churns	P7 / A	1/2b	II	17
2000	Cardoon extract	A/ A	4b	V	15
2000	Towel	A / A	1/2b	XV	6
2000	Cheese washing machine	A / A	1/2b	II	17
2000	Cheese brine	A / A	4b	I	14
2004	Castelo Branco Cheese, rind	A / A	4b	IV-1	11
2004	Castelo Branco Cheese, inside	A / A	4b	IV-1	11 (1)
2004	Castelo Branco Cheese, inside	A / A	4b	IV-1	11 (1)
2004	Cheese washing machine, zone 2	A / A	4b	IV-1	11 (1)
2004	Raw milk from an individual ewe	P2 / A	4b	IV-1	11 (1)
2004	Raw milk from an individual ewe	P2 / A	4b	IV-1	11 (1)

NT – Not typeable; ND – Not determined.

3.3. Evaluation of virulence

The mean log PFA virulence results obtained, ranged between 5.31±0.19 and 6.62±0.06 (Table 3). Log 3.34 was the low virulence cut-off threshold determined by Roche *et al.* (2001) and values above that should be considered as results of virulent potential. Our results for EGDe virulent reference strain were similar to those reported by Roche *et al.* (2001) and for this reason strains with values higher than 6.32±0.34 were considered as significantly high virulent ($P<0.05$). AFLP type II PFGE 17 strains obtained from milk of different producers (P6, P7) were not significantly different from each other. Three milk isolates (P2, P6, and P7) presented high values of PFA but the strain with the highest value was AFLP type V, PFGE 16, an isolate obtained at milk reception directly from churns of producer P5. The cheese isolate AFLP type V, PFGE 3 was significantly less virulent.

Table 3. Virulence potential of isolates of *L. monocytogenes* evaluated on HT29 monolayer cells**.**

Source	Serotype	AFLP type	PFGE type	Virulence (mean ± SD)*
EGDe, clinical	1/2a	ND	ND	6.32 ± 0,34
Cheese, A	4b	V	3	5,90 ± 0.18
Milk, P4	4b	I	14	5,52 ± 0.16
Milk, P5	4b	V	16	6,62 ± 0.06
Milk, P6	1/2b	II	17	6,08 ± 0.18
Milk, P7	1/2b	II	17	5,84 ± 0.04
Towel, A	1/2b	XV	6	5,31 ± 0.19
Milk, P2	4b	IV-1	11	6,02 ± 0.32

ND – Not determinated; PFA- plaque forming assay; * Log numbers of plaques for 10^7 CFU deposited. Values of mean log PFA ± standard deviation are from at least two independent experiments performed in duplicate.

4. Discussion

In this survey *L. monocytogenes* was recovered from one of the two main cheese production regions in the central part of Portugal, and is consistent to previously noted contamination in products from one of these regions (Leite *et al*., 2006). There was some evidence for the recurrence of specific strains within these cheese manufacturing environments in Portugal although most isolates from the same site differed. Recurrent isolation of individual strains of *L. monocytogenes* has been reported in particular food production environments, for example, in a dairy (Unnerstad *et al.*, 1996), as well as ice cream and smoked fish manufacturing environments (Miettinen *et al.,* 1999; Vogel *et al.,* 2001). However, in this study there was also a great diversity of strains within the food production areas as well as in the final product. This suggests multiple and diverse routes of contamination including raw milk, as well as plant machinery and the production environments. In opposition to this, a great homology was reported for high prevalent 4b strains that were isolated from cheese and processing environment in small-scale dairies in Austria which source of contamination was related with low personnel hygienic standards (Wagner *et al*., 2006). This also means that doubled hygiene both on farm and dairies will need to be considered to control sources of contamination from sites within the cheese factories as well as in raw materials brought into the factories.

Our previous studies showed that the cheese washing machines, together with the brine solution are microbiological hazard points in the environment of this cheese process (Pintado *et al*., 2005; Leite *et al*., 2006) and special care should be given to their control and sanitation. In addition, contamination of milk prior to receipt in the cheese manufacturing plant is also a point to be aware of and standards should be established for flock control at the farms. Guide lines must be given about quality and safety control at raw milk reception from different producers, on a legal national basis, to prevent microbiological hazards entrance. Strains of *L. monocytogenes* isolated from the environment of plant A were likely to be introduced into the premises by the contamination of the incoming milk at source, what might explain the elimination and recontamination cycles reported by Chambel *et al*., (2007). The introduction of *L. monocytogenes* through raw material is likely, especially since the same strain of *L. monocytogenes* with virulent potential, was detected in final product

leaving the cheese factory A in 2004. These contaminated cheeses were not sent for retail sale and were destroyed at the end of this study. The study of plant A showed sub-optimal cleaning and sanitation practices which were improved and applied as routine procedure ever since. These were targeted at all sites where the contamination was detected and resulted in the eradication of *L. monocytogenes* from the environment in the milking season of 2005 despite a similar level of microbiological surveillance.

Contamination of raw milk results from either the plant, machinery or other environmental sites in the dairy, from ovine fecal contamination during milking or from organisms being secreted into the milk as a result of mastitis. Ovine mastitis was identified on milk suppliers referred in this study but the results of more detailed observations and their relevance in the safety of this kind of cheese will be presented in further detail elsewhere.

This study highlighted that the real problem is the urgent need for preventive actions on a legal national basis for flock control at the farms. Preventive actions must include the establishment of efficient *Listeria* monitoring that should be mandatory for all cheese producers to capture contamination on a very early stage. Co-operative A decided for risk awareness of the associated members and established staff and farmer's educational programs.

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CAPÍTULO IV

High nisin susceptibility of *Listeria* spp. wild-type strains isolated from dairies with traditional cheese preservation in Portugal

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High nisin susceptibility of *Listeria* spp. wild-type strains isolated from dairies with traditional cheese preservation in Portugal

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Running title: Nisin susceptibility of *Listeria* strains

Abstract

Aims: Nisin susceptibility of 219 *Listeria* spp. wild-type strains isolated from milk, cheese and cheese processing environment was examined. METHODS AND
Methods and Results: Minimum inhibitory concentrations (MIC) were evaluated by the agar incorporation method, on TSYEGA medium. The variability of nisin susceptibility was very low especially at pH 5.5, with 98% of isolates showing MIC values between 10 and 50 IU ml^{-1} at 37ºC. The increase of pH from 5.5 to 6.8 at 37ºC resulted in an increase in the MIC values, from 10 – 50 to 50 – 100 IU or higher than 100 IU ml^{-1} in 68% of isolates. An increase in temperature from 20ºC to 37ºC at pH 5.5 resulted in an increase on MIC values just in 8% of all isolates tested. Nisin spontaneous resistance was observed more frequently at pH 6.8 and 37ºC, at a frequency of 10^{-3} to 10^{-4}.
Conclusions: The majority of tested strains were able to grow at 10 IU nisin ml^{-1} but not at 50 IU ml^{-1} at pH 5.5, showing a very susceptible profile.
Significance and Impact of Study: The low pH (5.5) and low temperature (20ºC) offer better conditions to the inhibitory action of nisin when applied as hurdle method to the control of *L. monocytogenes* strains from dairies sources.

Key words: Nisin; MIC; *Listeria monocytogenes;* susceptibility

1. Introduction

In the last decade the possibility of using bio preservation by addition of bacteriocins or bacteriocin-producing starter cultures has received increased interest in the food industry, driven by consumer choice for natural foods (Loessner *et al*. 2003, Devlieghere *et al*. 2004). Particular attention has been given to a class I bacteriocin named nisin that is a small peptide, membrane-active, heat-stable with increased stability at low pH (McAuliffe *et al*. 2001), with general regarded as safe status (GRAS) by the Food and Drug Administration (Delves-Broughton 2005) and regulated for use in the EU (Anon. 1995).

Several workers have investigated the inhibitory effect of nisin on *Listeria* spp. but the minimum inhibitory concentrations (MIC) reported are difficult to compare because they use different assay conditions, use single or few strains in their research and all in all generated confusing results likely to be due to strain or isolate-specific variation and for this reason are of limited value (Benkerroum and Sandine 1988, Mazzotta and Montville 1997, Ukuku and Schelef 1997). There is just one study that attempted to compare larger number of strains in the same study, reported in the literature, as far as we known. They evaluated the sensitivity to nisin of a collection of 339 wild-type strains of *L. monocytogenes* isolated from meat and found that they are quite more sensitive than the reference strains studied and reported in the literature (Rasch and knøchel 1998). They emphasise the need for multiple strain test to understand differences in sensitivity and to determine the likelihood occurrence of naturally-tolerant or resistant strains. To understand the variations observed on the effect of different concentrations of nisin at different pH and temperatures, again Ukuku and Schelef (1997) pointed in their studies, conducted with six strains of *L. monocytogenes*, the importance to examine a wide variety of strains to draw conclusions.

Concern regarding the use of bacteriocins has been linked with the development of resistant or highly tolerant strains of *Listeria* but studies have been conducted with a reduced number or culture collections reference strains (Ming and

Daeschel 1993, Davies and Adams 1994, Jydegaard *et al.* 2000). The occurrence of naturally-resistant food isolated stains is relevant from the preservation point of view.

The aims of this study were to examine (i) the variation in nisin susceptibility amongst a large number of *Listeria* spp. strains, at three different conditions (pH 5.5 and 20ºC; pH 5.5 and 37ºC; pH 6.8 and 37ºC), and (ii) in a comprehensive way the likely occurrence of natural resistance to nisin in wild-type strains isolated from milk, cheese and cheese processing environment.

2. Material and methods

2.1. Nisin and culture media

Nisin NP (5 x 10^6 IU g^{-1} potency) was a gift by Danisco Beaminster Ltd., U.K.. Stock solutions of nisin were prepared aseptically in HCl, 0.02 mol l^{-1}, divided into aliquots and stored at – 20ºC.

Tryptone Soy Yeast Extract Glucose Broth (TSYEGB, which contained: 30 g l^{-1} Tryptone Soya Broth; 3 g l^{-1} Yeast Extract; 10 g l^{-1} Dextrose, all OXOID, U.K.) and Bacto Tryptose Phosphate Broth (TPB, Difco) were used to prepare inocula of *Listeria* spp. cultures. Tryptone Soy Yeast Extract Glucose Agar (TSYEGA), which contained 15 g l^{-1} Agar (OXOID, U.K.) more than TSYEGB, was used for the determination of MIC of nisin. The pH was adjusted with HCl, so that after the addition of a solution of nisin, the final pH was 5.5 and 6.8. Culture dilutions used to prepare inocula were made in Peptone Salt Dilution Fluid (PSDF), according to ICMSF (1978). Tryptone Soya Agar (TSA, OXOID, U.K.) was used to determine bacteria counts. Trypticase Soy Broth (TSB) from BBL, Becton Dickinson and Company (NJ, USA), containing 10% (v/v) of glycerol (Sigma Chemical Co., St. Louis, MO) was used to maintain the bacterial cultures at – 80ºC by cryopreservation.

2.2. *Listeria* spp. strains

Evaluation of the susceptibility to nisin was done towards a total of 219 *Listeria* spp. strains, isolated over a period of 10 years, and previously described and

characterized (Pintado *et al*. 2005, Pintado *et al.* 2007). The strains comprise 141 *L. monocytogenes* and 78 *L. innocua*, which were isolated from Portuguese ripened cheeses (n = 110 isolates), raw ewes milk used to make cheese (n = 46 isolates) and cheese processing environment (n = 63 isolates), including cheese brine and cheese working surface samples. *L. monocytogenes* NCTC 11994 (serotype 4b, soft cheese isolate), was used as reference strain and the *L. monocytogenes* CP6 strain was used in the evaluation of nisin effect in a broth medium culture. This one is a cheese isolate, serogroup 4, associated with an ovine subclinical mastitis and cheese contamination (Pintado *et al.* 2009).

2.3. Minimum inhibitory concentration

The MIC evaluations against *Listeria* spp. were done by the agar incorporation method, as described by Ferreira and Lund (1996). Before use, two successive cultures of *Listeria* spp. were grown in TPB at 30ºC, for 24 h and for 16 - 18 h. The second culture was diluted in PSDF to give a suspension containing approximately 10^6 cfu ml^{-1}. Plates of TSYEGA, adjusted at pH 5.5 and 6.8, were prepared containing a given concentration of nisin (5, 10, 50, 100 and 200 IU ml^{-1}) and 10 μl of the inoculum (10^4 cells per drop) were inoculated on the nisin-containing agar plates by using the spot method. Inoculated nisin-free plates and plates with nisin but not inoculated were also incubated and used as control. After incubation at 37ºC for 24 h or 20ºC for 48 h, growth was evaluated visually and recorded as normal (confluent growth, similar to control without nisin), with spontaneous resistant (development of a few colonies, between 1 and 20) or no growth (total absence of the growth). MIC values were recorded as between the lowest concentration that gave complete absence of growth and the highest concentration that allowed growth. Frequency of nisin spontaneous resistance was also evaluated. Each assay was done in triplicate, on separate plates.

2.4. Effect of nisin on *L. monocytogenes* CP6 in broth medium

The TSYEGB medium (10 ml), adjusted to pH 5.5 and 6.8 with HCl, was used with final concentration of 50 IU ml^{-1} nisin and without nisin. Two successive cultures of *L. monocytogenes* CP6 were grown in TPB at 30ºC for 24 h and 18 h. The second culture was prepared to give a suspension of approximately 10^6 cfu ml^{-1} after

the inoculation of the TSYEGB media, and incubated at 20ºC and 37ºC. Viable bacteria were determined by plate count on TSA at 6, 24, 72 and 144 h, after incubation at 37ºC for 24 h. A negative control was done to test the sterility of the medium.

3. Results

The majority of tested strains, including the NCTC 11994 reference strain, were able to grow at 10 IU nisin ml^{-1} but not at 50 IU ml^{-1}, at pH 5.5, showing a very susceptible profile. The variability of *Listeria* spp. susceptibility to nisin was very low especially at pH 5.5, with 98% (214 / 219) of all isolates studied showing MIC values between 10 and 50 IU ml^{-1} at 37ºC and 92% (202 / 219) at 20ºC (Fig. 1).

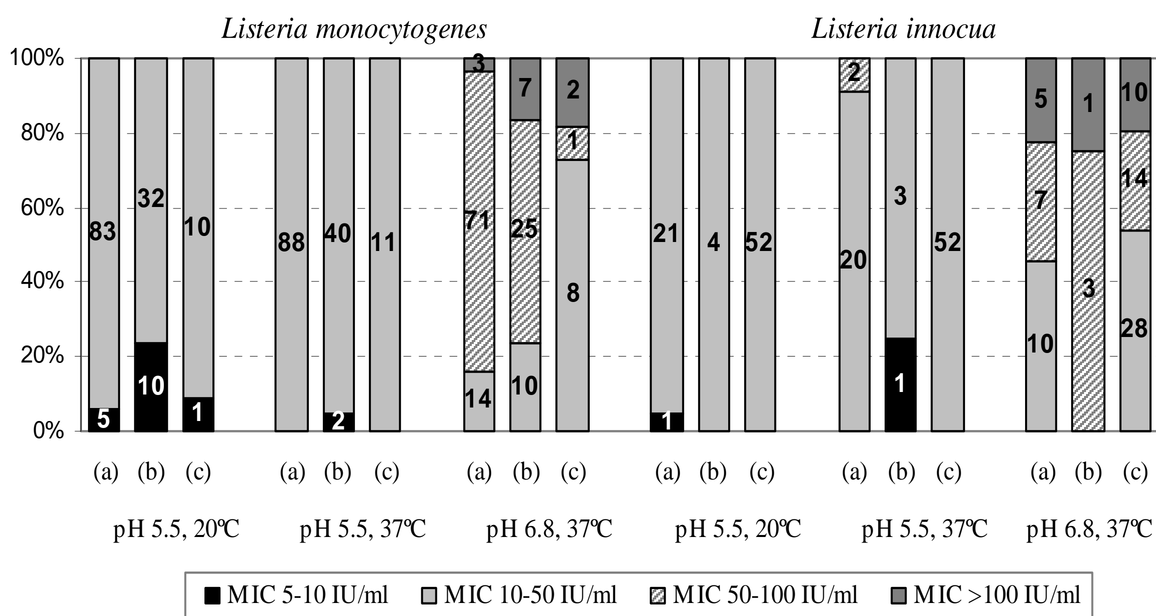

Figure 1. Susceptibility of *L. monocytogenes* (n = 141) and *L. innocua* (n = 78) strains to nisin (at 5, 10, 50, 100 IU ml^{-1}) on TSYEGA medium set at pH 5.5 and 6.8, and incubated at 20ºC and 37ºC. *Listeria* spp. strains were isolated from cheese **(a)**, milk **(b)** and environment **(c)** samples.

When the assay was done on agar adjusted at pH 6.8 and 37ºC, the susceptibility to nisin decrease and only 32% (70 / 219) of all isolates tested gave a MIC of 10 - 50 IU ml^{-1}, with the remaining 68% of isolates showing MIC values of 50 - 100 IU ml^{-1} or higher than 100 IU ml^{-1}. At pH 5.5 and 20ºC, about 8% of *Listeria* strains showed a more susceptible profile, with a MIC value of 5 - 10 IUml^{-1}. The reduction of temperature of incubation from 37ºC to 20ºC at pH 5.5 resulted in some reduction in the MICs.

No difference in susceptibility to nisin was found in respect to *L. monocytogenes* and *L. innocua* strains tested at pH 5.5. Nevertheless, when the pH was 6.8, *L. monocytogenes* strains showed a more susceptible profile than *L. innocua* strains. A higher percentage of isolates with the MIC 5 - 10 IU ml^{-1} of nisin was found within milk isolates in comparison with cheese isolates tested at pH 5.5 and 20ºC.

Nisin spontaneous resistance was observed more frequently at pH 6.8 and 37ºC than at other conditions tested (Tab. 1), and a frequency of 10^{-3} to 10^{-4} was calculated under these conditions of test.

The presence of 50 IU ml^{-1} of nisin in TSYGB medium resulted in death of the *L. monocytogenes* CP6 more quickly at 37ºC than at 20ºC. No re-growth was observed throughout the 144 hours essayed (Fig. 2). The *L. monocytogenes* CP6 grew well without nisin at all assay conditions, increasing the population of about 2 - 3 log within the first 24 h, but after that the bacterium growth decrease with a reduction rate more pronounced in case of 37ºC. After 144 h, a higher number of survivors was achieved at 20ºC and pH 5.5. In broth medium, this bacterium is more susceptible at 37ºC than at 20ºC. No survivors were found after 6 h at 37ºC and pH 6.8. At 20ºC the reduction of growth rate was not so pronounced as at 37ºC and it took longer to reach zero survivors at 20ºC than at 37ºC. Bouttefroy *et al.* (2000) also found a maximum inhibitory effect of nisin (50 IU ml^{-1}), in a modified TSYEB, at pH 6.6, when pH was tested from 5.0 to 8.2.

Table 1. Number of *Listeria* spp. strains that show nisin spontaneous resistant, tested at 0, 5, 10, 50, 100 and 200 IU ml^{-1}, on TSYEGA medium at different temperature and pH conditions.

Isolates	Nisin IU ml^{-1}	Spontaneous resistant pH5.5 20ºC	Spontaneous resistant pH5.5 37ºC	Spontaneous resistant pH6.8 37ºC
Cheese isolates (n = 110)	0	-	-	-
	5	-	-	-
	10	1	2	-
	50	-	-	44
	100	-	-	10
	200	-	-	-
Milk isolates (n = 46)	0	-	-	-
	5	-	-	-
	10	-	-	-
	50	-	-	9
	100	-	-	2
Environmental isolates (n = 53)	0	-	-	-
	5	-	-	-
	10	5	-	-
	50	-	-	11
	100	-	-	9
Total isolates (n = 219)	0	-	-	-
	5	-	-	-
	10	11	2	-
	50	-	-	67
	100	-	-	21

(-) No nisin spontaneous resistants were found.

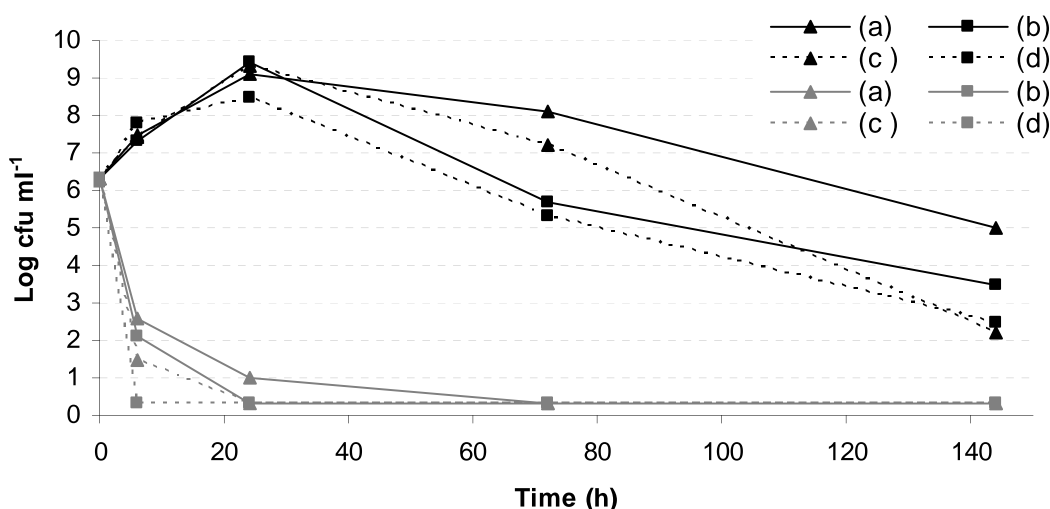

Figure 2. Growth of *L. monocytogenes* CP6 in TSYGB medium without nisin (black lines) and with 50 IU ml^{-1} of nisin (grey lines) at 20ºC and pH 5.5 **(a)**, 20ºC and pH 6.8 **(b)**, 37ºC and pH 5.5 **(c)**, and 37ºC and pH 6.8 **(d)**. Viable counts were determined from the means of experiments performed in triplicate.

4. Discussion

Several workers have investigated the inhibitory effect of nisin on *Listeria* spp. but the minimum inhibitory concentrations (MIC) reported have great variations. In this study, the strains tested showed a high susceptible profile (MIC range of 10 - 50 IU ml^{-1}) (Fig. 1), when compared with previous reports in the literature. Ferreira and Lund (1996) reported MIC values 10 to 20 fold higher. As their assay conditions were similar to ours regarding media and culture conditions, we concluded that the higher susceptibility found within these isolates of dairy origin evaluated in this study was isolate or strain specific, and probably may be related with its origin. Furthermore, we found a very low variability in susceptibility to nisin, despite the differences on provenance and isolation date of strains, with the most sensitive strains showing MIC values range of 5 – 10 IU ml^{-1} and the less sensitivity strains a MIC range of 10 – 50 IU ml^{-1}, at pH 5.5 and 20ºC. Isolates predominantly of *L. innocua* specie with environment origin showed similar susceptibility profile (10 – 50 IU ml^{-1}) at the three conditions assayed. Ferreira and

Lund (1996), evaluating 31 *Listeria* spp. strains from different provenances (associated with listeriosis outbreaks, references strains, and isolated from cheese, raw milk and salad vegetables), that were obtained from several laboratories culture collections, observed a greater variability that ranged from below 5 to 400 – 600 IU ml^{-1}, at pH 5.5 and 20ºC. Lower differences in MIC ranges, very similar to our values, were found by Rasch and Knøchel (1998) using a collection of 381 *L. monocytogenes*, that included 340 wild-type strains isolated from meat products. They inoculated the cultures by the spot method onto a nisin - tryptic soya agar (pH 6.6) supplemented with 0.2% Tween-80 and the plates were incubated at 30ºC during 16 h.

An increase in temperature of 17ºC (from 20ºC to 37ºC) at pH 5.5 resulted in an increase on MIC values just in 8% of all isolates tested: from 5 – 10 IU to 10 – 50 IU ml^{-1} for milk (n = 8), cheese (n = 6) and cheese brine solution (n = 1) strains of *L. monocytogene*s, and from 10 – 50 to 50 – 100 IU ml^{-1} for cheese isolates (n = 2) of *L. innocua* strains. The remaining 92% of isolates tested maintained the same nisin susceptibility profile at 20ºC and at 37ºC. Sensibility to nisin is generally reported to decrease with the increase of temperature (Thomas and Wimpenny 1996), despite the fact that nisin polypeptide was reported for having an optimum molecular stability at 37ºC (Rollema *et al.* 1995). Nevertheless, in this study a no negligible effect of temperature was only observed within 7 out of 46 milk isolates, 6 out of 110 cheese isolates and 1 out 10 cheese brine isolate.

We were expecting a lower inhibitory effect of nisin at pH 6.8 than at 5.5 with 37ºC as was reported before (Ferreira and Lund 1996), because the solubility of nisin is strongly dependent on pH, with highest solubility and inhibitory effect observed at acidic pH (Rollema *et al.* 1995). In this study, the increase of pH from 5.5 to 6.8 at 37ºC resulted in an increase in the MIC values, from 10 – 50 IU ml^{-1} to 50 – 100 or higher than 100 IU ml^{-1}, which means a decrease in inhibitory capacity of nisin, but that happened just in 68% (149 / 219) of all isolates under study, and the remaining 32% of the isolates maintained the same susceptibility profile at both pH values.

There was only two isolates with a MIC range of 50 – 100 IU ml^{-1}, when incubated at pH 5.5 and 37ºC, being all the others 217 isolates a MIC range of 10 – 50 IU ml^{-1}. These two isolates belong to *L. innocua* specie, were isolated from 2

different cheese samples from the same producer and demonstrated a negative catalase test. At pH 6.8 and 37ºC had MIC value higher than 100 IU ml^{-1} and at pH 5.5 and 37ºC they were the most tolerant strains studied.

No difference in susceptibility to nisin was found with respect to species tested at pH 5.5 and 37ºC, but at pH 5.5 and 20ºC and at pH 6.8 some *L. monocytogenes* isolates were more susceptible than *L. innocua* strains. Ferreira and Lund (1996) observed that differences in susceptibility to nisin were not correlated with difference in serotype and that strains of *L. innocua* were as resistant as the most resistant *L. monocytogenes*.

We were expecting lower nisin susceptibility profiles for cheese isolates than for isolates from milk and environment, as the result of the selection done by the growth of nisin-producing lactic acid bacteria cultures, naturally present in this kind of cheeses. However, in general nisin susceptibility of cheese isolates was not different from nisin susceptibility of milk isolates, unless a higher percentage of isolates with the MIC 5 - 10 IU ml^{-1} of nisin found within milk isolates (22%, 10 / 46) in comparison with 5% (6 / 110) within cheese isolates tested at pH 5.5 and 20ºC. The most frequent MIC value found for environment isolates at 37ºC and pH 6.8 was 10 – 50 IU ml^{-1}, lower than for milk and cheese isolates (50 – 100 IU ml^{-1}).

The results obtained with growth in broth medium are in contrast with MIC results, since the *L. monocytogenes* CP6 was inhibited at pH 6.8 and 37ºC after 6 h and were need 72 h to reach complete inhibition at pH 5.5 and 20ºC. These results are likely related with the common phenomenon of autolysis when *Listeria* strains, growing in broth medium, enter in the stationary phase of growth (Jones *et al.*, 1995), than are linked with the nisin activity. They reported that this phenomenon is enhanced under anaerobic growth conditions at 37ºC and when glucose is the sole carbon source. At 20ºC, the reduction of growth rate was not so pronounced as at 37ºC and it took longer to reach zero survivors at 20ºC than at 37ºC. Bouttefroy *et al.* (2000) also found a maximum inhibitory effect of nisin (50 IU ml^{-1}), in a modified TSYEB, at pH 6.6, when pH was tested from 5.0 to 8.2.

In the present study, nisin spontaneous tolerance was observed more frequently at pH 6.8 and 37ºC than at other conditions tested (Tab. 1) and a frequency of 10^{-3} to 10^{-4} was calculated. These data are in agreement with those reported by Gravesen *et al.* (2002), since they found that nisin resistance frequency

was considerably reduced at environmental stress in the form of low temperature (10ºC), reduced pH (5.5), or presence of NaCl (6.5%). Moreover, no clear correlation could be established for differences observed on growth at low pH and two salt concentrations tested, when two wild-type strains isolated from soft cheese and their derivate nisin-resistant phenotype were compared, in one study (Martinez *et al.* 2005). Gravesen *et al.* (2002) found that stability of nisin resistance is variable. Although the spontaneous resistant were not stable, they are of great concern as they could grow in the food. The problem of high tolerance may be minimized by the use of nisin in association with other preservation methods or with other food preservatives, as low pH.

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CAPÍTULO V

Properties of whey protein based films containing organic acids and nisin to control *Listeria monocytogenes*

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Properties of whey protein based films containing organic acids and nisin to control *Listeria monocytogenes*

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Running title: Antilisterial whey protein films

Abstract

Whey protein isolate and glycerol were mixed to form a matrix to incorporate antimicrobial agents and produce edible films with antimicrobial activity against *Listeria monocytogenes* strains isolated from cheeses. Various organic acids were used to decrease pH down to approximately 3. In a preliminary assay without nisin, the effect of each organic acid was evaluated with respect to the rheological properties of the film solutions and the inhibitory and mechanical properties of the films. Lactic, malic, and citric acids (3%, wt/vol), which were used in a subsequent study of their combined inhibitory effect with nisin (50 IU/ml), had significantly higher antilisterial activity (P<0.05) compared with the control (2 N HCl, 3% [wt/vol] with nisin). The largest mean zone of inhibition was 4.00 ± 0.92 mm, for malic acid with nisin. Under small-amplitude oscillatory stress, the protein-glycerol-acid film solutions exhibited a predominantly viscous behavior or a weak gel behavior, with the storage modulus (G´) slightly higher than the loss modulus (G´´). The malic acid–based solution was the only one whose viscosity was not influenced by the addition of nisin. The addition of nisin resulted in a nonsignificant (P>0.05) increase in the percentage elongation at break. Results from tensile and puncture stress were variable, but in general not significant differences were found after the incorporation

of nisin. The overall results support the use of malic acid with nisin to produce effective antimicrobial films to control *L. monocytogenes* growth.

Key words: Whey protein antimicrobial films, *Listeria monocytogenes,* nisin, organic acids

1. Introduction

The use of bioactive films to wrap and protect cheese is common in the industry. The most frequently used coatings probably are those based on polyvinyl acetate and polyvinyl alcohol, but the safety of these products has been discussed (*9*). Films based on whey protein isolate (WPI) are a promising alternative because this material is edible, has high chemical affinity for the cheese surface, and is a by-product of the cheese industry with a high impact as a pollutant waste. Approximately 10^8 kg/year of liquid whey is produced in Portugal, therefore its use needs to be promoted for environmental reasons.

The best protocol for making whey protein films, as studied by McHugh et al. (*11*), is 10% (wt/wt) aqueous protein solution at neutral pH heated for 30 min at 90ºC. The heat denaturation is required to open the globular structure of the whey proteins, break the disulfide bonds, and form new intermolecular disulfide and hydrophobic bonds, resulting in a water-insoluble whey protein–based film (*11*). Because the combination of resulting intermolecular disulfide bonds and intermolecular interactions between protein chains based on hydrogen bonding, hydrophobic interactions, and electrostatic forces produces brittle films (*17*), plasticizers must be added. These additives overcome brittleness by reduction of protein chain-to-chain interaction and an increase of polymer-plasticizer hydrogen bonds.

Nisin is an amphiphilic polypeptide produced by certain strains of *Lactococcus lactis* subsp. *lactis*. The antimicrobial activity of nisin inhibits a wide range of gram-positive bacteria (*6*). A double mode of action of nisin was reported by Ryan et al. (*16*). Nisin binds to lipid II, inhibiting cell wall synthesis and forming pores in the cytoplasmic membrane. As a natural food preservative, nisin is currently permitted in the European Union according to Directive 95/2/EC (*2*) at the levels of

12.5 mg/kg in ripened cheese and processed cheese. Although formation of WPI films is favored in more alkaline filmogenic solutions (*3*), most antimicrobial agents, such as nisin, are more effective under acidic conditions. The stability, solubility, and activity of nisin are greatest at low pH (*15*). Like nisin, organic acids have antimicrobial activity and are classified as generally recognized as safe, compatible with their use in the development of an edible antimicrobial coating.

The purpose of the work reported here was to study and characterize the solutions and the films made with WPI as a biopolymer, glycerol as plasticizer, and various organic acids with and without nisin as antimicrobial agents to control *Listeria monocytogenes*. An understanding of the relative influence of various acidulants and nisin on whey protein–based film properties will allow researchers to select a formulation that provides the most effective film for wrapping and protecting cheeses.

2. Materials and methods

2.1. Preparation of film solutions and films

Seven grams of WPI (92% minimum protein content, dry basis; Carbery Group, Ballineen, Ireland) was dissolved and homogenized in 100 ml of distilled water and stirred with a magnetic bar for 15 min, and 3.0 g of glycerol (Sigma-Aldrich Chemical Co., St. Louis, MO) was introduced to the solution and stirred again for 15 min. Each organic acid (acetic acid, Merck, Darmstadt, Germany; malic acid, BDH Chemicals Ltd., Poole, UK; citric, ascorbic, and formic acids, Panreac, Barcelona, Spain; lactic acid, Riedel-de-Haën, Seelze, Germany; fumaric acid, Sigma-Aldrich), at 1.5 and 3.0% (wt/vol), was added, and the final solution was stirred for 15 min until uniform solutions were obtained. As a pH control, 3.0% (wt/vol) 2 N HCl (Riedel-de-Haën) was used. After measuring the pH, the solutions were heated at 90ºC for 30 min in a shaking water bath. Once cooled at room temperature, an adequate volume of nisin solution at 10^5 IU/ml (nisin NP, Danisco, Beaminster, UK) previously made with 20 mM HCl was incorporated to produce a final concentration of 50 IU/ml. This concentration was previously determined as the lowest concentration tested that gave complete growth inhibition of *L. monocytogenes*

strains used in this assay (data not shown). Films were cast by aseptically pipetting 9 ml of solution into 90 mm polystyrene disposable plates. After drying for 24 to 48 h at 23 ± 2ºC and 50 ± 5% relative humidity, the films were peeled from the plates and stored under the same controlled conditions until used.

2.2. Rheological measurements

The rheological measurements were performed in a controlled-stress rheometer (RS75, Haake, Karlsruhe, Germany) at 20 ± 0.1ºC, using serrated parallel plate geometry (PP35 or PP60) to overcome the slip effect and a gap of 1.000 mm. Oscillatory tests (mechanical spectra) were carried out using a frequency range of 0.01 to 100 Hz and a stress value in the linear viscoelastic region for each solution (1 to 20 Pa). Steady-state flow measurements were also made, and viscosity versus shear stress curves were constructed using a logarithmic ramp of stresses increasing from 0.001 Pa to 2,300 Pa. In all cases, at least two measurements were made.

2.3. Mechanical properties of films

Tensile strength was measured with a TA-XTplus Texture Analyser (Stable Micro Systems, Godalming, UK). The films were cut into strips 70 mm long and 20 mm wide and selected for lack of defects, and the film thickness was measured with a micrometer with a sensitivity of 0.01 mm (Absolute Digimatic Calliper, Mitutoyo Ltd, Andover, UK) at six or more random locations to obtain the mean thickness. These film strips were mounted in the film-extension grips, tensile grips A/TG, of the texture analyzer and stretched at a speed of 0.8 mm/s in tension mode. Tensile stress (in MPa) was calculated as the ratio of the peak load developed during the test by the film cross-sectional area (sample width times sample thickness). The percentage of elongation at break was calculated as the ratio of the extension of the specimen at the moment of rupture to the initial gage length, multiplied by 100. At least 15 measurements were taken. The puncture strength of the films was measured on a texturometer TA-XT2 (Stable Micro Systems) by mounting square film strips (30 by 30 mm) on a specially designed base with a hole 9.89 mm diameter. With a cylindrical 2-mm probe and a test speed of 2.0 mm/s in compression mode, the films were punctured through the hole and the force (in Newtons) at the point of rupture was recorded; this value divided by the probe contact area was expressed as the

puncture stress. The percentage of elongation at puncture also was calculated. All mechanical tests were performed at least 15 times at 50 ± 5% relative humidity and 23 ± 2 ºC.

2.4. Culture media and bacterial strains

Nine strains of *L. monocytogenes* previously described as isolated from raw ewe's milk cheeses (*12, 13*) and two reference strains, *L. monocytogenes* NCTC 11994 (serotype 4b, soft cheese isolate) and *Listeria innocua* NCTC 11288 (serotype 6a), were used. For all these strains, the MIC values for nisin were 10 to 50 IU/ml at pH 5.5 and 50 to 100 IU/ml at pH 6.8. The cultures were maintained at -80ºC in BBL Trypticase soy broth (Becton Dickinson, Sparks, MD) containing 10% (vol/vol) glycerol and subcultured twice at 37ºC 18 to 24 h before use in tryptone soy broth with yeast extract and glucose (TSBYEG), which contained 30 g/liter tryptone soya broth, 3 g/liter yeast extract, and 10 g/liter dextrose (Oxoid, Basingstoke, UK).

2.5. Antimicrobial agar diffusion assay

Films were aseptically cut into discs (6 mm diameter) and placed on the surface of plates of Trypticase soy agar containing yeast extract and glucose (TSAYEG), which contained 15 g/liter agar (Oxoid) more than TSBYEG, adjusted to pH 6.2 with HCl. The plates were previously inoculated with a swab of an 18-h culture of each test strain. After 24 h of incubation at 37ºC, the zones of the inhibition formed around the film discs were measured with the same micrometer used to measure film thickness. The assay was done in triplicate.

2.6. Statistical analysis

Data were analyzed with version 14.0 of the SPSS program (SPSS Inc., Chicago, IL). The Scheffe´ multiple range test ($P<0.05$) was used to detect differences between mean values of film properties.

3. Results and discussion

3.1. Selection of type and concentration of organic acids

WPI-glycerol (Gly) solutions and films made with 1.5 and 3.0% (wt/vol) concentrations of several organic acids were tested. With citric, acetic, malic, formic, ascorbic, fumaric, and lactic acids at 1.5 and 3.0%, the mean (±SD) pH of the resulting solutions was 3.23 ± 0.43 (Table 1).

Table 1. Effect of various organic acids on pH, first Newtonian limiting viscosity, storage modulus, loss modulus and loss tangent of the whey protein isolate film solutions plasticized with glycerol.

Acid (%, wt/vol)	pH	Visual analysis	Viscosity (η_0) (Pa.s)[a]	G´(Pa) Storage modulus (f=0.01Hz)	G´´ (Pa) Loss modulus (f=0.01Hz)	Tan (δ) (G´´ / G´)
Acetic (1.5%) +Lactic (1.5%)	3.27	Viscous	73.09 ± 19.43	$6.4x10^{-1}$	$5.4\ x10^{-1}$	$8.5\ x10^{-1}$
Lactic, 1.5%	3.34	Fluid	16.41 ± 23.04	$1.9\ x10^{-5}$	$3.4\ x10^{-3}$	$1.8\ x10^{2}$
Lactic, 3.0%	2.92	Viscous	650.00 ± 43.13	$1.2\ x10^{0}$	$9.4\ x10^{-1}$	$7.8\ x10^{-1}$
Malic, 1.5%	3.18	Fluid	23.65 ± 17.18	$6.3\ x10^{-2}$	$1.4\ x10^{-1}$	$2.2\ x10^{0}$
Malic, 3.0%	2.76	Fluid	0.20 ± 0.01	$3.0\ x10^{-5}$	$5.1\ x10^{-3}$	$1.7\ x10^{2}$
Citric, 1.5%	3.37	Viscous	5622.29 ± 378.25	$1.8\ x10^{1}$	$7.8\ x10^{0}$	$4.4\ x10^{-1}$
Citric, 3.0%	2.90	Viscous	42.16 ± 2.88	$3.0\ x10^{-1}$	$2.6\ x10^{-1}$	$8.7\ x10^{-1}$
Formic, 1.5%	2.78	Viscous	0.99 ± 0.15	$2.5\ x10^{-5}$	$8.3\ x10^{-3}$	$3.3\ x10^{2}$
Formic, 3.0%	2.48	Viscous	8.07 ± 0.86	$5.9\ x10^{-2}$	$1.2\ x10^{-1}$	$2.0\ x10^{0}$
Acetic, 1.5%	3.88	Viscous	ND[b]	ND	ND	ND
Acetic, 3.0%	3.58	Viscous	ND	ND	ND	ND
Fumaric, 1.5%	3.21	Viscous	ND	ND	ND	ND
Fumaric, 3.0%	3.11	Viscous	ND	ND	ND	ND
Ascorbic, 1.5%	4.01	Viscous	ND	ND	ND	ND
Ascorbic, 3.0%	3.66	Fluid	ND	ND	ND	ND

[a] Values are means ± SD (n ≥ 2); [b] ND, not determined.

Because acids become very weak when the target pH is lower than the pKa value, citric and malic acids (with pKa values of 3.13 and 3.40, respectively) were most effective at lower pH values (around 3) than were acetic acid (pKa of 4.76) and ascorbic acid (pKa of 4.17).

Despite the incorporation of 3% glycerol as a plasticizer, films made with 1.5 and 3.0% formic acid, 1.5 and 3.0% acetic acid, 1.5 and 3.0% fumaric acid, 1.5% ascorbic acid, and 1.5% citric acid were not viable because of extreme brittleness (Table 2), so these types of films were eliminated from the study. The WPI-Gly films with 3.0% ascorbic acid had a brown color and also were eliminated.

Some influence of the nature of the acid molecule on the mechanical properties of the films was expected. Cagri et al. (*5*) reported that organic acids might have a plasticizing effect because they are small molecules with hydroxyl groups. Among the acids studied, citric, malic, and lactic acids have four, three, and two hydroxyl groups, respectively, and acetic and formic acids have only one hydroxyl group each. Hydroxyl groups of the plasticizer replace polymer-polymer interactions by developing polymer plasticizer hydrogen bonds, which probably explains the higher elongation values for the films produced with citric and malic acids (Table 2).

Table 2. Effect of various organic acids on mechanical properties, visual aspects, and thickness of low-pH whey protein isolate films plasticized with glycerol.[a]

Acid (%, wt/vol)	Aspect	Tensile stress (MPa)	Elongation at break (%)	Puncture stress (MPa)	Elongation at puncture (%)	Thickness (μm)
Acetic (1.5%) + Lactic (1.5%)	Extensible	0.51 ± 0.23 [A]	2.22 ± 1.05 [AB]	0.59 ± 0.20 [AB]	1.97 ± 0.28 [B]	96 ± 9 [A]
Lactic, 1.5%	Extensible	1.85 ± 0.87 [CD]	1.21 ± 0.45 [A]	0.75 ± 0.18 [BC]	2.07 ± 0.36 [B]	130 ± 27 [D]
Lactic, 3.0%	Extensible	1.32 ± 0.22 [BC]	3.10 ± 0.74 [B]	0.83 ± 0.17 [C]	1.05 ± 0.27 [A]	127 ± 18 [CD]
Malic, 1.5%	Extensible	1.89 ± 0.85 [CD]	2.08 ± 0.65 [AB]	0.64 ± 0.14 [ABC]	2.10 ± 0.46 [B]	109 ± 14 [ABC]
Malic, 3.0%	Extensible	1.19 ± 0.18 [B]	9.03 ± 2.86 [C]	0.50 ± 0.20 [A]	5.52 ± 0.74 [D]	125 ± 11 [BCD]
Citric, 1.5%	Brittle	ND[b]	ND	ND	ND	ND
Citric, 3.0%	Extensible	1.96 ± 0.21 [D]	8.3 ± 1.09 [C]	0.76 ± 0.14 [BC]	3.72 ± 0.31 [C]	106 ± 9 [AB]

[a] Values are means ± SD (n ≥ 15); Within a column, means with different letters are significantly different ($P < 0.05$, Scheffé test); [b]ND, not determined (brittle films).

With 1.5% acetic acid and 1.5% ascorbic acid, the whey proteins started to precipitate, and the solutions became white and opaque, forming thick gels that could not be spread to form films. All the other films were transparent. The pH values of the WPI-Gly film solutions of those two acids were 3.88 and 4.01, respectively (Table 1), close to the isoelectric point (pI) of two major constituents of the whey protein (β-lactoglobulin and α-lactalbumin; isoelectric points of 5.2 and 4.5 to 4.8, respectively). The resulting gel was more rigid, in contrast to the more spreadable solution obtained when the pH of the solutions was far from the pI, corresponding to the pH of minimum water-holding capacity and low solubility (*1*). Electrostatic interactions around the pI are responsible for protein aggregation, and consequently the proteins are less flexible and therefore less prone to form a cohesive film (*7*). As reported by De Wit (*7*), we found that WPI-Gly film solutions with pH values lower than 3.5 were completely transparent.

Under small-amplitude oscillatory stress, the WPI-Gly-acid film solutions exhibited a predominantly viscous behavior or a weak gel behavior with G´ slightly higher than G´´ (Table 1). When the concentration of organic acids increased from 1.5 to 3.0%, an increase of apparent viscosity with lactic and formic acids and a decrease with malic and citric acids were observed. These results are in agreement with the changes seen in the storage modulus (G´) and loss modulus (G´´) in the oscillatory stress testing. This finding was attributed to the different number of hydroxyl groups from the acids, which interfere with the protein matrix by introducing hydrogen bridging, thus increasing flexibility and decreasing structure and hence viscosity. The overall results of apparent viscosity ranged from 0.20 ± 0.01 Pa.s for 3.0% malic acid–based film solutions and to 5,622.29 ± 378.25 Pa.s for 1.5% citric acid–based film solutions (Table 1).

According to Resch et al. (*14*), β-lactoglobulin gels formed at pH < pI have weak mechanical properties, associated with inhibition of disulfide bonding, compared with those of gels formed at pH > pI, which exhibit strong elastic properties. Nevertheless, we observed a wide range of behavior according to the type and concentration of the organic acid incorporated into the WPI-Gly solutions, all with pH < pI. These variations in the rheological properties, which were observed when different organic acids interacted with whey proteins in the film matrix, are not easily understood because they depend on temperature, pH, ionic strength, whey

protein concentration, and the nature and concentration of other solids (*7*). Because the temperature and the concentrations of the whey protein and glycerol were the same, the variations in the rheological properties are likely the result of differences in the ionic strength of the solutions, the nature of organic acid, and pH.

The results of mechanical analysis ranged from 0.51 ± 0.23 to 1.96 ± 0.21 MPa for tensile stress and 0.50 ± 0.20 to 0.83 ± 0.17 MPa for puncture stress (Table 2). These values for the low-pH films are substantially lower than that reported previously for high-pH films (*5*). A low-pH environment would likely prevent disulfide bond formation in the protein matrix, thereby weakening the film structure. Our results for tensile stress are similar to those reported by Sothornvit et al. (*18*); however, the results for elongation percentage are 10-fold lower. In general, an association between an increase in elongation percentage and a decrease in tensile stress was observed with the increase of organic acid concentration from 1.5 to 3.0%.

The study on the effect of various organic acids (at 1.5 and 3.0%) on antimicrobial properties of whey protein films revealed that the films made with 1.5% citric acid and 1.5 and 3.0% formic acid had no zones of inhibition on the agar plates; antimicrobial activity was evident only directly under the disc, although the pH of the WPI-Gly–formic acid solutions tended to be lower. The films made with 3% malic, lactic, and citric acid (with 3.67-, 2.51-, and 2.89-mm zones of inhibition, respectively) had greater inhibitory effects than did films with the same acids at 1.5% (1.83-, 0.50-, and 0.0-mm zones of inhibition, respectively).

According to the overall results, WPI-Gly film solutions acidified with malic, lactic, and citric acids at 3.0% (wt/vol) were selected for evaluation in a subsequent assay with and without the lantibiotic nisin, and these results were compared with those for WPI-Gly film solutions made with 3.0% (wt/vol) 2 N HCl (pH 3.13) as the pH control.

3.2. Antimicrobial properties of films made with acids (3.0%) and nisin

The incorporation of 50 IU/ml nisin in WPI-Gly films acidified with malic, lactic, and citric acids resulted in a significant increase ($P < 0.05$) in the zones of inhibition, indicating a synergistic antimicrobial effect of nisin plus malic or citric acid (Fig. 1) when applied to *L. monocytogenes* and *L. innocua* strains on TSAYEG at pH 6.2, the usual pH of the rind of Castelo Branco cheese (*13*). The enhanced

effect could be due to fact that the citric, malic, and lactic acids can take advantage of the pores previously opened by the nisin molecule and can penetrate more easily into the bacterial cell membrane. In addition, citric, lactic, and malic acids have chelating activity (*10*), and the performance of nisin can be improved by the effect of these chelators. Solubility of nisin is strongly dependent on pH and ionic strength, with highest solubility observed at low pH and low ionic strength (*15*). At 37ºC, the temperature of incubation used in our assay, optimal molecular stability was recorded at pH 3.

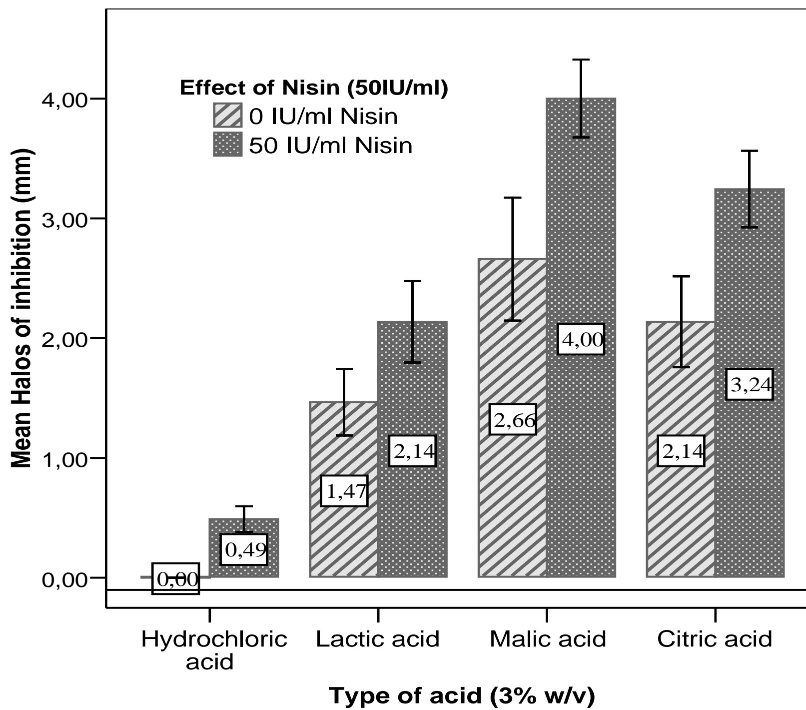

Figure 1. Antimicrobial activity of WPI-Gly films made with organic acids (3%) and nisin (50 IU/ml) against strains of *Listeria monocytogenes* (n=10) and *L. innocua* (n=1). The bars represent average triplicate values (n=33) for the zones of inhibition on TSAYEG (pH 6.2), with 95% confidence intervals. No inhibition of growth was observed around or under the discs prepared with 2N HCl (3%) without nisin.

Antimicrobial activity of films with nisin plus malic, lactic, and citric acids were significantly different ($P < 0.05$) from the control with nisin alone. No inhibition under or around the 3% HCl discs without nisin (pH 3.13) was observed, in contrast with a clear zone of inhibition around the organic acid discs.

Organic acids with low molecular weight have higher antimicrobial activity. Nevertheless, in this study lactic acid had the lowest killing effect, followed by citric acid and malic acid. Citric and malic acids had the greatest inhibitory effect probably because the pKa of these acids is lower than the pKa of lactic acid, and consequently at similar concentrations, citric and malic acids tend to decrease the pH more than does lactic acid. The greater inhibitory effect also likely reflects the fact that the reduced ability of citric and malic acids to enter bacterial cells is compensated for by their enhanced ability to dissociate inside the cell and thus decrease internal pH (*19*). Citrate and malate ions have been reported to chelate polyvalent cations essential to microbial growth. Buchanan and Golden (*4*) observed that antimicrobial effects of malic acid against *L. monocytogenes* were similar to those of citric acid in brain heart infusion broth.

The highest average of zone of inhibition was 4.00 ± 0.92 mm, which was obtained using the combination of malic acid plus nisin. The next largest zone was obtained with citric acid plus nisin (3.24 ± 0.90 mm). Eswaranandam et al. *(8)* also reported that malic and citric acids had greater antilisterial activity than did lactic acid when these acids were used at 2.6% in nisin-incorporated soy protein films with 0.9% glycerol.

3.3. Mechanical and rheological properties of film solutions made with acids (3.0%) and nisin

The stress range for linear viscoelasticity was determined by dynamic stress sweep at 1 Hz. Film solution made with citric acid had the most structured network (followed by film solutions made with lactic acid and malic acids) with an extended linear region (data not shown). The mechanical spectra of the WPI-Gly film solutions made with malic, citric, and lactic acids were somewhat reduced by nisin incorporation (Fig. 2). WPI-Gly–citric acid solution without nisin had very weak gel-like behavior, with G´ slightly higher than G´´. The steady shear measurements (Fig.

3) were consistent with previous findings; this solution had stronger shear thinning behavior and a value of the first limiting viscosity closer to 10^3 Pa.s.

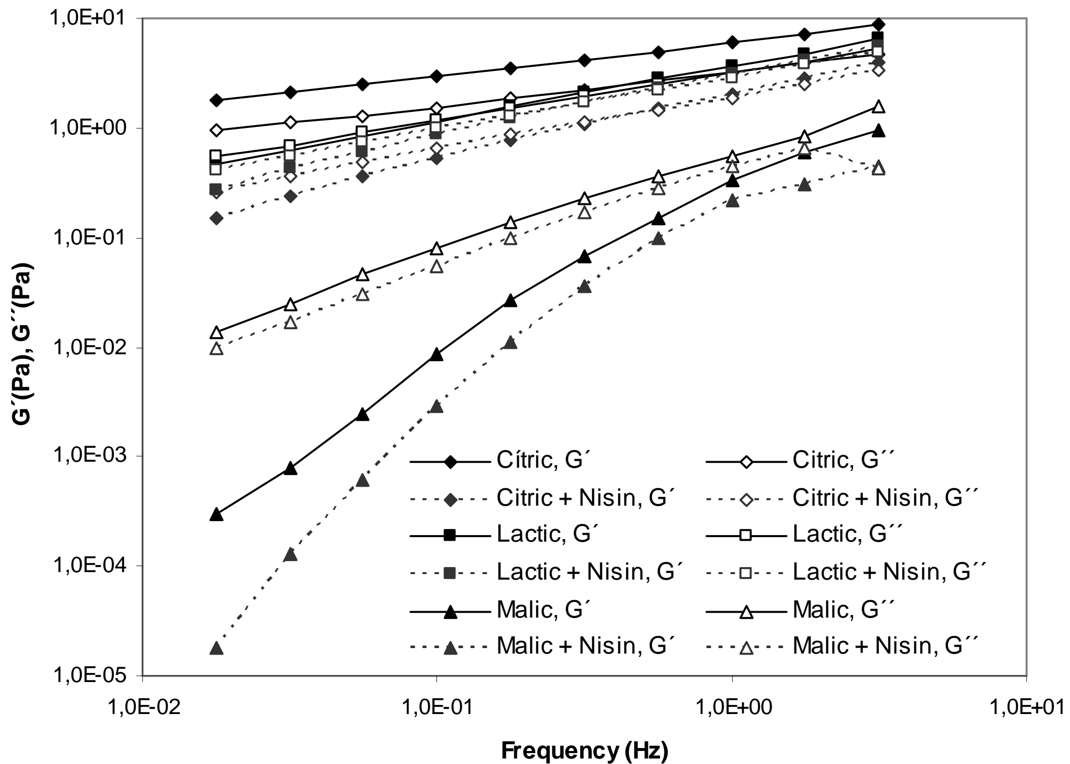

Figure 2. Mechanical spectra (G´ and G´´ versus frequency) at 20ºC of the WPI-Gly film solutions (pH 3) acidified with 3.0% (wt/vol) citric, malic, and lactic acids without and with 50 IU/ml nisin.

The whey protein solutions studied are non-Newtonian fluids. The viscosity (η_0) of the solutions was reduced by the addition of nisin for solutions made with citric and lactic acids, but both solutions kept the shear thinning pattern. Nisin is a 3.4-kDa antimicrobial peptide composed of 34 amino acids with hydrophobic properties (*6*) and disulfide and free thiol bonds. The lower viscosity observed after incorporation of nisin in the protein network may be due to competition for intermolecular bonds between whey-whey protein chains and whey-nisin protein chains, resulting in a reduction of interactions between the whey protein chains. High viscosities are unfavorable during preparation of films because it is more difficult to disperse the ingredients and eliminate air bubbles, which cause irregularities in the

dried films. Thus, films made with malic acid had the best performance because they had a low apparent viscosity that was not reduced by the addition of nisin.

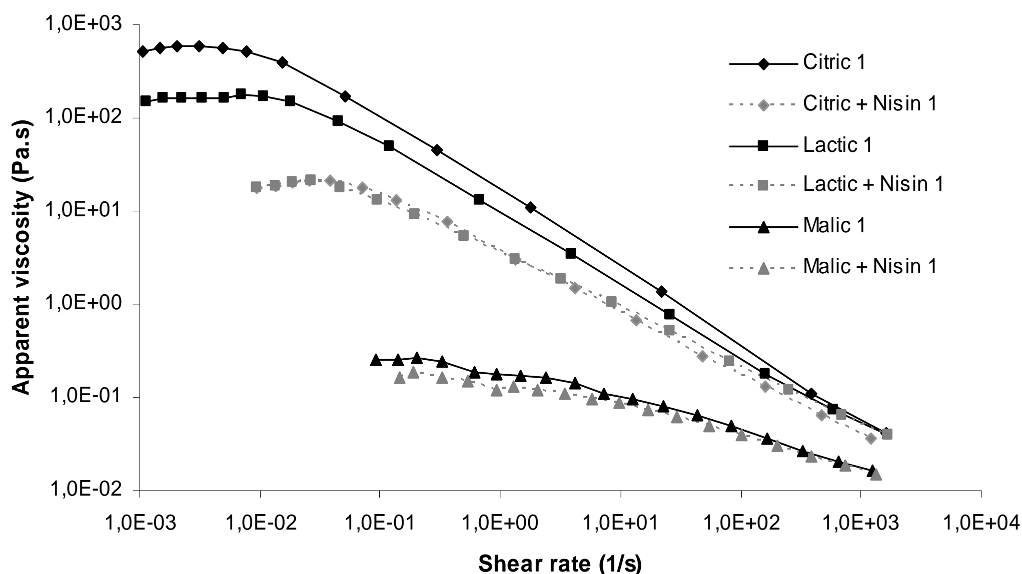

Figure 3. Apparent viscosity as a function of shear rate duplicates at 20ºC for film solutions produced from whey protein isolate and glycerol acidified with various organic acids at 3.0% (wt/vol) without and with 50 IU/ml nisin.

The incorporation of nisin resulted in a significant increase ($P < 0.05$) in elongation percentage in films made with malic and citric acids (Table 3). Results from tensile and puncture stress tests were variable, but in general we did not found significant differences after the incorporation of nisin.

Table 3. Mechanical properties and thickness of low-pH whey protein isolate films with and without nisin.[a]

Type of film[b]	Tensile stress (MPa)	Elongation at break (%)	Puncture stress (MPa)	Elongation at puncture (%)	Thickness (μm)
2N HCl	3.18 ± 0.49 [C]	1.14 ± 0.23 [A]	0.34 ± 0.09 [BC]	0.64 ± 0.12 [A]	95 ±10
2N HCl + Nisin	4.16 ± 0.40 [D]	1.88 ± 0.25 [A]	0.31 ± 0.08 [AB]	0.75 ± 0.17 [A]	90 ±5
Lactic acid	2.20 ± 0.24 [B]	6.23 ± 1.32 [B]	0.44 ± 0.08 [CD]	2.66 ± 0.19 [B]	92 ±8
Lactic acid + Nisin	2.11 ± 0.20 [B]	7.59 ± 0.79 [BC]	0.27 ± 0.04 [AB]	2.27 ± 0.22 [B]	86 ±6
Malic acid	1.16 ± 0.11 [A]	8.87 ± 1.15 [C]	0.23 ± 0.03 [A]	3.99 ± 0.43 [C]	108 ±15
Malic acid + Nisin	1.38 ± 0.21 [A]	9.01 ± 0.98 [C]	0.28 ± 0.06 [AB]	4.86 ± 0.53 [D]	97 ±8
Citric acid	2.24 ± 0.29 [B]	7.01 ± 1.38 [BC]	0.47 ± 0.07 [D]	4.13 ± 0.41 [C]	107 ±18
Citric acid + Nisin	2.31 ± 0.35 [B]	7.24 ± 0.93 [BC]	0.46 ± 0.08 [D]	4.68 ± 0.47 [D]	82 ±9

[a] Values are means ± SD (n ≥15). Within a column, means with different letters are significantly different ($P < 0.05$). [b] All acid concentrations were 3% (wt/vol), and all nisin concentration were 50 IU/ml.

In conclusion, various interactions between organic acids and whey proteins within the films or film solutions resulted in variations in the antimicrobial, rheological, and mechanical properties. Nisin (50 IU/ml) improved the antilisterial effects of lactic, citric, and malic acids (3.0%, wt/vol) when used in whey protein films plasticized with glycerol (7.0% WPI and 3.0% Gly, wt/vol) against *L. monocytogenes* strains isolated from semi-soft raw ewe's milk cheese in a disc diffusion assay on TSAYEG at pH 6.2. Films made with malic acid plus nisin had the highest antimicrobial activity, and the rheological, mechanical, and antimicrobial properties indicate that these edible films could be used to wrap cheese and inhibit the growth of this pathogen, improving food safety.

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CAPÍTULO VI

Control of pathogenic and spoilage microorganisms from cheese surface by whey protein films containing malic acid, nisin and natamycin

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Control of pathogenic and spoilage microorganisms from cheese surface by whey protein films containing malic acid, nisin and natamycin

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Abstract

The inhibitory effects of nisin, natamycin and malic acid, incorporated in whey protein films with pH 3, were investigated alone or with addition of sucrose esters, Tween80 or EDTA. Water vapour permeability measurements and mechanical and rheological tests were also assessed. EDTA and Tween 80 did not significantly (P<0.05) influence the inhibitory activity of films against *Pseudomonas aeruginosa* and *Yarrowia lipolytica* in contrast with the improved effect against *Listeria monocytogenes*, *Penicillium commune* and *Penicillium chrysogenum*. Sucrose esters reduced significantly (P<0.05) the inhibitory effect for *Y. lipolytica* and *Penicillium* spp.. The present work provides an antimicrobial film formulation with potential to be a hurdle against spoilage and pathogenic microorganisms isolated from cheese surface.

Key words: Antimicrobial films, cheese safety, *Listeria monocytogenes,* rheology of films

1. Introduction

Cheese is a ready-to-eat food easily contaminated on the surface by undesirable microorganisms. Some are spoilage microorganisms which may produce uncharacteristic visual appearance and diminish the commercial value of the cheeses, such as *Yarrowia lipolytica*, *Pseudomonas aeruginosa* and *Penicillium* spp. but others are pathogenic such as *Listeria monocytogenes*, which have been associated with foodborne listeriosis by consumption of cheese (McLauchlin, Mitchell, Smerdon, & Jewell, 2004). The Gram-negative bacteria *Pseudomanas* spp. are the most important of the psychrotrophs that dominate the microflora of raw milk (Sorhaug & Stepaniak, 1997). Strains of *Ps. aeruginosa* have been associated with undesirable browning reactions on cheese rind (Ogunnariwo & Hamilton-Miller, 1975), and some are phatogenic. The *Y. lipolytica* yeast, frequently found in cheeses, was also reported to be associated with browning phenomenon (Carreira, Paloma, & Loureiro, 1998). *Penicillium* is the genera of moulds most frequently isolated from naturally contaminated cheese rind samples and include mycotoxigenic strains. All these microorganisms comprise strains with psychrotrophic characteristics that could increase in number during cold storage (Sorhaug & Stepaniak, 1997).

Most recently, the food industry showed an increasing interest in antimicrobial edible films to enhance food safety and product shelf life. Different matrix can be used to incorporate antimicrobial agents (Appendini & Hotchkiss, 2002), including whey protein isolate (WPI). In general, the resistance to water vapour transmission of protein films is limited because they are highly polar polymers with a high level of hydrogen bonding and hydroxyl groups (Ko, Janes, Hettiarachchy, & Johnson, 2001). Furthermore, in high humidity environments the water vapour barrier properties are subsequently reduced because of protein films´ susceptibility to moisture absorption and swelling. This attribute could be detrimental when foods coated by these films are submitted to high humidity storage conditions, increasing the diffusion coefficient of antimicrobial agents to the food from the film matrix. Therefore, the incorporation of agents such as fatty acid esters to decrease water vapour permeability (WVP) is necessary. Also, the type and concentration of plasticizer, such as glycerol and sorbitol, influences the ability of films to attract water.

Nisin is a hydrophobic and cationic polypeptide, a food-grade preservative that exhibits antimicrobial activity towards a wide range of Gram positive bacteria but shows little or no activity against Gram negative bacteria, yeasts, and moulds (Delves-Broughton, 2005). It has been observed that Gram-negative cells, normally insensitive to the action of nisin, can be sensitized by the addition of chelating agents, such as EDTA, which disrupt the integrity of the outer membrane and allow the bacteriocin to access the cytoplasmic membrane (Boziaris & Adams, 1999; Stevens, Sheldon, Klapes, & Klaenhammer, 1992;). Sucrose esters of fatty acids and Tween 80, used commonly as food emulsifiers, are nonionic emulsifiers known to inhibit the growth of a wide range of microorganisms, primarily against Gram-positive bacteria and fungi, when used alone or in combination with an antimicrobial agent as nisin (Thomas, Davies, Delves-Broughton, & Wimpenny, 1998) or organic acids (Monk, Beuchat, & Hathcox, 1996). Natamycin is a polyene natural antimycotic with a wide range of antimicrobial spectrum against yeasts and moulds (Welscher, Napel, Balagué, Souza, Riezman, et al., 2008). Natamycin incorporating coat solutions are used by cheese industry but the commercial available products are in general based on polyvinyl acetate or polyvinyl alcohol, which food safety iniquity has been discussed (EFSA, 2005). This is of great concern because there are cheese consumers that appreciate cheese as a whole, consuming the inner and the rind of the cheeses.

The objectives of this work were to study how the incorporation of different emulsifier, plasticizer and chelator agents into WPI – based films with malic acid, nisin and natamycin interfere with the antimicrobial activity against *L. monocytogenes*, *Ps. aeruginosa, Penicillium* spp. and *Y. lipolytica* and the mechanical and WVP properties of the films.

2. Materials and methods

2.1. Materials used to produce films and specifications

Whey protein isolate (with a protein content of 92% minimum, dry basis) was kindly supplied by Carbery Food Ingredients, Ballineen, Co. Cork, Ireland. The sucrose esters of fatty acids were a gift from Mitsubishi-Kagaku Foods Corporation,Tokyo, Japan (Ryoto Sugar Ester S-970 with 31-33% sucrose

monostearate and 20-22% sucrose distearate, HLB-value 9) and from Sisterna B.V., BH Roosendaal, The Netherlands (SP30, nonionic emulsifier, sucrose distearate, HLB-value approx. 6, 30% monoester content; and SP50, nonionic emulsifier, sucrose stearate, 50% monoester content, HLB-value approx. 11). Nisin NP ($5x10^6$ IU/g potency), Nisaplin (minimum 10^3 IU/mg of Nisin A and minimum 50% NaCl), and Natamax Salt were a gift from Danisco Beaminster Ltd., Beaminster, U.K. Glycerol and D-Sorbitol were purchased from Sigma Chemical Co., St. Louis (MO), USA; Hydrochloric acid from Riedel-de-Haën, Seelz, Germany; DL-Malic acid from BDH Chemicals Ltd.; EDTA from Aldrich, St. Louis (MO), USA; and Tween80 from Acroyali , Quingdao, China.

2.2. Microbial cultures and media

Twelve different microorganisms (Table 1) were used, which included six isolated from the rind of Castelo Branco Cheese, a semi-soft ripened ewe´s cheese from the central part of Portugal (Pintado, Oliveira, Pampulha, & Ferreira, 2005). The P2 and P6 strains of *Ps. aeruginosa*, and the moulds *Penicillium chrysogenum* and *Penicillium commune,* were isolated using selective agar media: Pseudomonas Aeromonas Selective Agar Base acc. to Kielwein (GSP Agar; Merck KGaA, Darmstadt, Germany) and Potato Dextrose Agar medium (PDA; from Oxoid, UK), respectively. *Pseudomonas* spp. strains were purified and identified to the species level using API® 20NE (bioMérieux® SA, France). *Pseudomonas aeruginosa* ATCC 15692 was kindly supplied by Instituto Superior Técnico, Technical University of Lisbon, and was used as reference strain.

All the *Pseudomonas* strains used in this study were maintained at 4ºC on slants of TSYEGA medium (l^{-1}): 30g Tryptone Soy Broth (Biokar Diagnostics, Beauvais, France), 6g Yeast Extract (Oxoid), 10g Glucose (COPAM, Portugal), and 18g Agar (Dário Correia, Portugal), with the pH adjusted to 6.2 with HCl. The *L. monocytogenes* strains were maintained at –80ºC in Trypticase™ Soy Broth (TSB; Becton, Dickinson and Company) containing 10% (v/v) Glycerol, and subcultured twice in TSYEGB medium (containing the same components as TSYEGA, without Agar), at 37ºC through 24 h and 18 h before use. Strains of *Y. lipolytica* were maintained at 4ºC on slants of GYPA medium which comprised 5.0g l^{-1} Peptone (Oxoid), 5.0g l^{-1} Yeast Extract (Oxoid), 2.0g l^{-1} Glucose (COPAM), and 20.0g l^{-1}

Agar (Dário Correia), with the pH adjusted to 6.2 with HCl. *Ps. aeruginosa* and *Y. lipolytica* strains were selected after assessing for the production of brown pigments on a Cheese-Tyrosine Agar medium (Carreira et al., 1998) containing (l^{-1}): 200g soft cheese, 40g NaCl (Merck), 10g L-tyrosine (Sigma), 12g agar, and pH adjusted to 7.0. All six strains produced brown pigmentation in this medium. Strains of *Penicillium* spp. were isolated from rind of ripened cheeses and identified to the genera level using the following media: Czapek Yeast Autolysate Agar (CYA), Creatine Sucrose Agar (CREA), Yeast Extract Sucrose Agar (YES) and Blakeslee Malt Extract Autolysate Agar (MEA), as recommended by Samson and Frisvad (2005). The strains were then sent to Centraalbureau voor Schimmelcultures (CBS, Fungal Biodiversity Centre, The Netherlands) for further identification. They were identified as *Penicillium chrysogenum* and *Penicillium commune*. *Penicillium roqueforti*, PRB6 HYP5D, CHOOZIT, is a cheese culture used as reference, and was kindly supplied by Danisco (Copenhagen, Denmark).

Table 1. Identification and source of microorganisms used in this study.

Microorganisms and references	Source of isolation
L. monocytogenes NCTC[a] 11994	---
L. monocytogenes CP6	Rind of ewe´s cheese
L. monocytogenes M12	Ewe´s cheese (Pintado et al., 2005)
Ps. aeruginosa ATCC[b] 15692	---
Ps. aeruginosa P2	Rind of ewe´s cheese
Ps. aeruginosa P6	Ewe´s raw milk
Y. lipolytica CBS[c] 6659	---
Y. lipolytica ISA 1668	Rind of ewe´s cheese (Carreira et al., 1998)
Y. lipolytica ISA 1708	Rind of ewe´s cheese (Carreira et al., 1998)
P. roqueforti (CHOOZIT™ PRB6 HYP5D)[d]	---
P. chrysogenum	Rind of ewe´s cheese
P. commune	Rind of ewe´s cheese

[a] National Collection of Type Cultures, London, United Kingdom.

[b] American Type Culture Collection, Manassas (VA), USA.

[c] Centraalbureau voor Schimmelcultures, Delft, the Netherlands.

[d] Danisco, Copenhagen, Denmark.

2.3. Preparation of film solutions and films

Seven grams of whey protein isolate were completely dissolved in distilled water (100 ml of total volume) by stirring for 15 min. Glycerol or sorbitol (1.5%, 2.25% and 3.0% w/v) were then incorporated and stirred for a further 15 min followed by the addition of malic acid (3.0% w/v) and stirred a further 15 min. After measuring the pH (pH Meter M82, Radiometer, Copenhagen), the solutions were heated at 90ºC for 30 min in a shaking water bath to form a film matrix by denaturation of protein. Once cooled at room temperature until approximately 50ºC, antimicrobial agents were incorporated into film solutions at final concentrations: Nisaplin® (50 IU nisin/ml), Natamax® Salt (0.002 g of natamycin/ml and 0.005 g of natamycin/ml), EDTA (0.1%, w/v), Tween80 (0.15%, w/v), sucrose distearate SP30 (0.075%, w/v), sucrose monostearateSP50 (0.075%, w/v) and sucrose monostearate S970 (0.075%, w/v). Film solutions were homogenised and 9ml were aseptically pipetted into 90mm diameter disposable plates. After drying for 24-48 h at 23 ± 2 ºC and 50 ± 5 % relative humidity (RH), the films were peeled out from the plates and stored under these conditions until use. Films were cut into 70 mm x 20 mm portions for mechanical measurements and aseptically cut into 6-mm-dia discs for inhibition assays.

2.4. Antimicrobial diffusion-type assay

The discs for the inhibition assay with antimicrobial agents were aseptically transferred to TSYEGA or GYPA plates, previously seeded with a swab of a 24 h culture of each organism given in Table 1, using the first medium for the bacteria and the second for the fungi. After an incubation of 24h at 37ºC for bacteria and 24 to 48h at 25ºC for yeasts and moulds, the width of the inhibition zone around each film disc was measured with a micrometer (Absolute Digimatic Calliper, Mitutoyo Ltd, Andover, UK), to the nearest 0.01 mm, and the assay was done in three independent trials. Two different assays were done, the first for testing the interactions between nisin, malic acid and natamycin. The second assay was prepared aiming to test the incorporation of different agents to improve antimicrobial properties of the films.

2.5. Film thickness

Thickness of films was measured with a micrometer (Absolute Digimatic Calliper, Mitutoyo Ltd, Andover, UK; having a sensitivity of 0.01 mm) around the film testing area (70 x 20 mm^2) at six random locations and averaged. The determinations were done before WVP and mechanical analysis assays.

2.6. Water vapour permeability determination

Water vapour permeability of films was measured using the ASTM Standard Method E96-95 (ASTM, 1995), known as the "cup method", modified by McHugh, Avena-Bustillos, and Krochta (1993) to be applied to edible films. Circular film specimens of 3.2 cm diameter were sealed with silicon onto the mouth of small circular test glass cups with 5 ml of distilled water and placed inside an excicator with silica that was placed under the perforated platform where cups were placed. A fan set to generate an air flow of 180 rpm was placed above the cups to maintain moisture conditions at the film surface. An air gap of about 2.6 cm exists between the film and the surface of distilled water inside the cup. The temperature set condition was 16ºC and a thermo-hygrometer (HANNA Instruments) was introduced in the test chamber to record relative humidity (RH). The RH value inside the cups (100%) was higher than the RH outside the cups (1.8% to 11.3%). The weight loss of the cups was monitored over a 24 h period with intervals of at least 3 h between readings. The water vapour transmission rate was calculated by linear regression of the plotted weight loss slope as function of time and dividing by the exposed area of the films ($8.04 \times 10^{-4} m^2$). Permeance was calculated according to Gennadios, Weller, and Gooding (1994), taking in consideration the air gap resistance due to stagnant air layer between the underside of the film and the surface of distilled water contained in the cup. Water vapour permeability was calculated as the product of permeance and the average thickness of the film. All tests were done in triplicate.

2.7. Mechanical properties of films

Puncture strength of the films was measured on a texturometer TA-XT2 (Stable Micro System, UK) by mounting squared film strips 30 mm x 30 mm on a specially designed base with a hole of 9.89 mm diameter. With a cylindrical probe of 2 mm and a test speed of 2.0 mm/s in a compression mode, the films were punctured

through the hole and the distance (mm) travelled by the probe and the force (N) at the point of rupture was recorded, divided by probe area and expressed as puncture stress (PS). The percentage of elongation (%E) at puncture was also calculated. At least 15 measurements were done. All mechanical tests were performed at 50 ± 5% relative humidity and a temperature of 23 ± 2ºC.

2.8. Rheological measurements

The rheological measurements were performed in a controlled-stress rheometer (RS75; HAAKE, Germany), at 20 ± 0.1 ºC, using serrated parallel plate geometry (PP60), in order to overcome the slip effect, and a gap of 1.000 mm. Oscillatory tests (mechanical spectra) were carried out using a frequency range of 0.01-100 Hz and a stress values comprised in the linear viscoelastic region for each solution (1-2 Pa). Steady-state flow measurements were also done and viscosity versus shear stress curves were performed using a logarithmic ramp of stresses increasing in 17-30 min from 0.001 Pa to 2300 Pa. In all cases, at least two measurements were done. The rhelogical measurements were done on the film solutions based on whey protein isolate (7% w/v), malic acid (3% w/v), glycerol (3% w/v), with the incorporation of nisin (50 IU/ml) and/or natamycin (0.005 g/ml), with the purpose to study the influence of natamycin in viscosity and viscoelastic properties of the solutions.

2.9. Statistical analysis

Statistic for data analysis was done with version 14.0 and 16.0 of the SPSS program (SPSS Inc., Chicago, USA). Scheffé´s multiple range test (P<0.05) was used to detect differences in film properties mean values.

3. Results and discussion

3.1. Antimicrobial activity

Our previous work (Pintado, Sousa, & Ferreira, 2006) revealed that WPI (7.0% w/v) – based films with glycerol (3.0% w/v) as plasticizer, malic acid (3.0% w/v) as antimicrobial and acidifying agent, and nisin (50IU/ml) as antimicrobial agent are effective to control foodborne pathogenic *L. monocytogenes* strains isolated from cheese samples when tested in a disc-diffusion assay. Since the rind of cheeses have a wide range of other microorganisms, including bacteria, moulds and yeasts, and since nisin is an antimicrobial agent with efficacy only for Gram-positive bacteria, the introduction of the antimycotic agent natamycin in the whey protein isolate – glycerol – malic acid (WPI-G-MA) films was analysed. The results (Table 2) revealed that *Y. lipolytica* and *Penicillium* spp. strains were only inhibited by the incorporation of natamycin into the films, as we expected, and the zones of inhibition were independent of the acid used to lower the pH of the films to approximately 3.

Table 2. Zones of inhibition (mm) produced by low pH (3) antimicrobial films made with whey protein isolate, WPI (7.0% w/v), glycerol, G (3.0% w/v), acids (3.0% w/v) and a combination of different agents (nisin, 50IU/ml, and natamycin, 0.005g/ml), in an antimicrobial disc-diffusion assay using TSYEGA or GYPA medium at pH 6.2.

	Zones of inhibition (mm)			
Composition of WPI - G films	*Listeria monocytogenes* CP6	*Pseudomonas aeruginosa* P6	*Yarrowia lipolytica* ISA1708	*Penicillium roqueforti* PRB6 HYP5D
Malic acid	2.5	0.5	(-)	(-)
Malic acid + Natamycin	1.2	0.5	8.3	12.4
Malic acid + Nisin	3.2	0.8	(-)	(-)
Malic acid + Natamycin + Nisin	3.3	1.0	8.4	12.4
HCl 2N	(-)	0	(-)	(-)
HCl 2N+ Natamycin	(-)	0	8.0	11.9
HCl 2N + Nisin	0.6	0	(-)	(-)
HCl 2N + Natamycin + Nisin	0.6	0	8.0	11.6

(-) No inhibition under or around the film; 0 - Growth inhibition just under the film.

A higher inhibitory effect against *L. monocytogenes* was observed with malic acid in association with nisin. No inhibitory effect around the disc films was observed against *Ps. aeruginosa* strain when nisin with HCl 2N incorporated into WPI films were used. As *Ps. aeruginosa* is a Gram-negative bacterium, it is nisin-resistant because their outer membrane prevents the passage into the cell of large hydrophobic molecules such as nisin (Thomas et al., 1998). The results in Table 2 also showed that no differences existed in the zones of inhibition obtained with nisin alone or nisin plus natamycin against bacteria strains tested, and no differences existed in the zones of inhibition obtained with natamycin alone or natamycin plus nisin against moulds and yeasts strains tested. This suggests an independent action and differentiated targets of nisin and natamycin. Welscher et al. (2008) revealed that natamycin blocks fungal growth by binding specifically to ergosterol, present almost exclusively in the fungi plasma membranes, and not present in bacteria.

A selective antimicrobial activity was seen on the addition of sucrose esters SP30, SP50 and S970 to the whey protein isolate – malic acid – nisin - natamycin (WPI-MA-N-NA) films (Fig. 1).

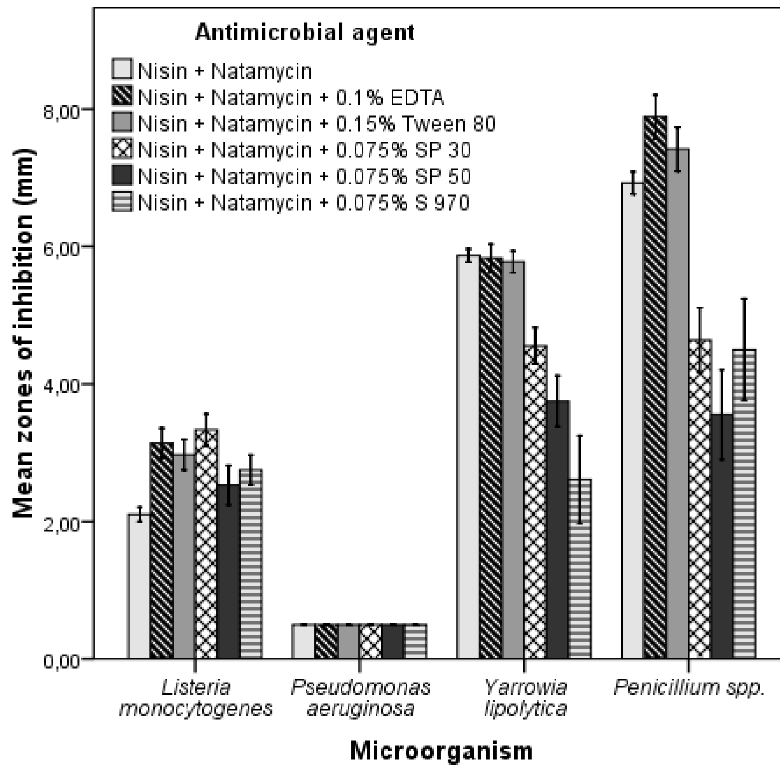

Fig. 1. Effect of the addition of 0.1% of EDTA, 0.15% of Tween80, and 0.075% of sucrose esters (SP30, SP50, S970) on antimicrobial activity of films prepared with whey protein isolate (7%), nisin (50 IU/ml), natamycin (0.002 g/ml) and sorbitol or glycerol (1.5%, 2.25%, 3.0%) as plasticizer, against strains of *L. monocytogenes*, *Ps. aeruginosa*, *Y. lipolytica* and *Penicillium* spp.. Error bars represent ± 1 standard error.

While the inhibitory effect was slightly improved for *L. monocytogenes* strains or was nonexistent for the *Ps. aeruginosa* strains, in the case of yeast and mould strains a considerable decrease in the inhibitory effect was observed when the sucrose esters were added. These results suggested an antagonistic interaction between sucrose esters and the other film components for the fungi growth control. A probable explanation is based on the capacity of sucrose esters to bind with ergosterol, diminishing the target site of natamycin. Despite the decrease of natamycin activity

observed, the antifungal action was not completely lost. An antimicrobial effect of sucrose fatty acid esters, alone or in association with nisin, against *L. monocytogenes* has been reported previously (Monk & Beuchat, 1995; Thomas et al., 1998). This work showed that this effect was not negligible when these agents were incorporated into a whey protein based film, with respect to *L. monocytogenes*.

All five agents studied improved the inhibitory activity of nisin and malic acid against *L. monocytogenes*. In opposition, none of these five agents enhanced the natamycin activity against *Y. lipolytica* and *Ps. aeruginosa* strains. Boziaris and Adams (1999) reported a poor chelating power of EDTA under acidic conditions, however a small enhanced inhibitory effect when EDTA and nisin were used against *L. monocytogenes* and *Penicillium* spp. strains was observed. This was also reported by Branen and Davidson (2003), with respect to *L. monocytogenes.* Treatment with metal-chelating agents as EDTA generally results in removal of divalent cations by chelation the lipopolysaccharide (LPS) layer of the outer membrane of Gram-negative bacteria, destabilizing its structure, thus increasing permeability (Vaara, 1992), and promoting the activity of other antimicrobials. There is a considerable variability of EDTA sensitivity for various species of the genera *Pseudomonas* and *Ps. aeruginosa* is considered to be EDTA-sensitive (Ayres, Furr, & Russel, 1999). Maybe the three strains of *Ps. aeruginosa* studied here have resistance mechanisms or the EDTA concentration was too low to produce any effect.

The effect of the agents tested on inhibition of *Ps. aeruginosa* strains in TSYGA medium showed that none was able to cause an increase of inhibition. Thomas et al. (1998) also reported no antimicrobial effect against Gram-negative bacteria when nisin was used alone or in combination with sucrose esters of fatty acids. In food protection, Gram-negative bacteria are of great concern due to their inherent resistance to some antimicrobials because of the protective action of the outer membrane of the cell wall (Belfiore, Castellano, & Vignolo, 2007).

Tween 80 (0.15%, w/v) did not enhance the inhibitory activity of nisin and natamycin against *Ps. aeruginosa* and *Y. lipolytica* but the antimicrobial effect was improved with the addition of Tween 80 against *L. monocytogenes* and *Penicillium* spp.

3.2. Viscosity and viscoelastic properties

Under SAOS, the mechanical spectra of the film solutions based on whey protein isolate (7% w/v), malic acid (3% w/v), glycerol (3% w/v) and nisin (50 IU/ml) exhibited a predominantly viscous behaviour, where the loss modulus was greater than the storage modulus (around 0.45 and 0.25, respectively). This behaviour was not modified by natamycin (0.005 g/ml) incorporation. Under steady shear, the limiting viscosity η_0 of first Newtonian plateau for the solutions tested showed to be unaffected by the addition of natamycin compared to solutions based on whey protein isolate, malic acid, glycerol and nisin, both with a shear thinning pattern and a first limiting viscosity η_0 value closer to 10^{-1} Pa.s. This fluid showed a low apparent viscosity, which is favourable to its industrial use in curtain or applications dip coating.

3.3. Water vapour permeability

The results show that an increase of concentration of glycerol and sorbitol from 1.5% to 3.0% resulted in an increase in WVP (from 1.55 to 2.75 g.mm/m^2.d.KPa for glycerol and from 1.11 to 2.34 g.mm/m^2.d.KPa for sorbitol) (Fig.2). Several authors had observed this behaviour (Irissin-Mangata, Bauduin, Boutevin, & Gontard, 2001; Maté & Krochta, 1996; Vanin, Sobral, Menegalli, Carvalho, & Habitante, 2005), which is usual in hygroscopic films, such as WPI protein films. Hydroxyl groups of the plasticizers replace polymer-polymer interactions by developing polymer-plasticizer hydrogen bonds, thereby increasing intermolecular spacing and permeability of film material (McHugh, Aujard, & Krochta, 1994). The increase of plasticizer concentration resulted in a linear relation between the amount of plasticizer and WVP. Maté and Krochta (1996) reported the same effect when WPI films plasticized with glycerol were studied.

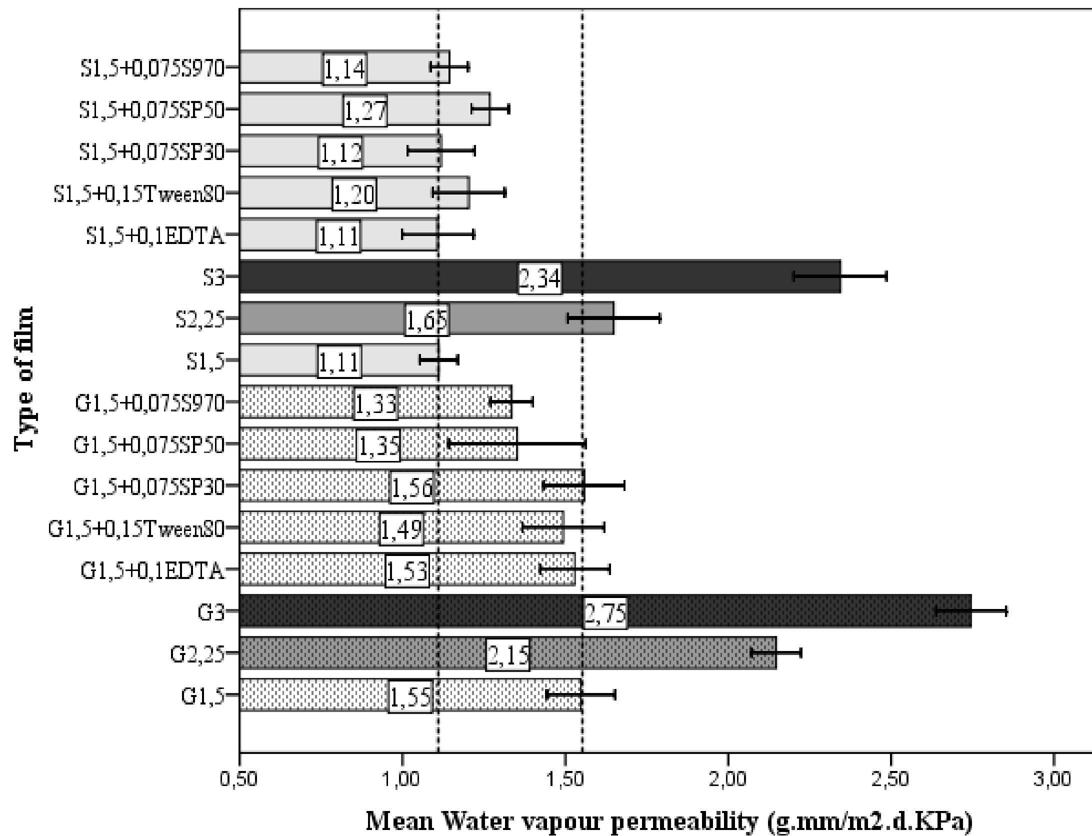

Fig. 2. Mean values of water vapour permeability (g.mm/m^2.d.KPa) for films produced with sorbitol (S) or glycerol (G) as plasticisers (at 1.5%, 2.25% and 3.0%), alone or with sucrose esters (SP30, SP50 and S970, all at 0.075%, w/v), EDTA (0.1%, w/v) or Tween80 (0.15%, w/v). All films are based on whey protein isolate (7.0%, w/v), malic acid (3.0%, w/v), nisin (50 IU/ml) and natamycin (0.002 g/ml). Error bars represent ± 1 standard error.

At equal concentrations, sorbitol (S)-plasticized whey protein films, exhibited significantly (P<0.05) lower WVP than films with glycerol (G), maybe due to the fact that glycerol is more polar, more soluble and is a smaller molecule than sorbitol. Kim and Ustunol (2001) reported that glycerol is more hygroscopic than sorbitol.

The water vapour permeability was not affected by the incorporation of sucrose esters (S970, SP30 or SP50), EDTA or Tween80 on WPI-plasticized (1.5%) films (Fig.2).

3.4. Mechanical properties

In general, glycerol-WPI films had higher % elongation (%E) and lower puncture stress (PS) than sorbitol-WPI films. Higher values of %E for glycerol-WPI films are probably attributed to the fact that these films contain more plasticizer per mol than those containing sorbitol, since the molecular weight of sorbitol is twice than that of glycerol (182 and 92 of Mw, respectively). Several authors had observed a molecular weight effect of the plasticizer on the mechanical properties (Sothornvit & Krochta, 2001; Sothornvit, Olsen, McHugh, & Krochta, 2007). On the other hand, the glycerol molecules possess a very high dielectric constant, a higher polarity in solution and are more hygroscopic than sorbitol.

Sucrose esters, EDTA or Tween80 increased significantly ($P<0.05$) the PS and the %E of WPI-glycerol (1.5%) films (Table 3). Regarding WPI-sorbitol (1.5%) films, the addition of sucrose esters, EDTA or Tween80 increased the PS but had no significant effect on %E.

Glycerol (3.0%)-WPI films showed significantly ($P<0.05$) lower puncture stress than the other formulations. Values of PS showed the most consistent dependence on plasticizer content while %E relationship to plasticizer content was somewhat variable.

Table 3. Measured mechanical properties (puncture stress and % of elongation) for composite films based on whey protein isolate, WPI (7.0% w/v), malic acid, MA (3.0% w/v), nisin, N (50 IU/ml) and natamycin, NA (0.002 g/ml), prepared with different agents (EDTA, Tween80, and sucrose esters: SP30, SP50 and S970) and glycerol or sorbitol at different concentrations.

Agents incorporated in WPI-MA-N-NA films	Puncture stress (MPa)		% Elongation	
	Mean ± SD	SE	Mean ± SD	SE
1.5% Glycerol	0.690 ± 0.122 ^{bc}	0.030	4.10 ± 0.35 ^{ab}	0.09
2.25% Glycerol	0.692 ± 0.160 ^{bc}	0.036	5.99 ± 1.06 ^{e}	0.24
3.0% Glycerol	0.391 ± 0.465 ^{a}	0.010	5.46 ± 0.92 ^{cde}	0.21
1.5% Glycerol + 0.1% EDTA	0.895 ± 0.271^{cd}	0.078	4.26 ± 0.37 ^{abc}	1.11
1.5% Glycerol + 0.15% Tween 80	1.010 ± 0.175 ^{defg}	0.039	5.64 ± 0.66 ^{de}	0.15
1.5% Glycerol + 0.075% SP30	0.906 ± 0.116 ^{cde}	0.025	5.16 ± 0.66 ^{bcde}	0.15
1.5% Glycerol + 0.075% SP50	0.983 ± 0.121 ^{def}	0.027	5.02 ± 0.38 ^{bcde}	0.08
1.5% Glycerol + 0.075% S970	1.108 ± 0.143 ^{defg}	0.045	5.05 ± 0.37 ^{bcde}	0.12
1.5% Sorbitol	0.910 ± 0.066 ^{cde}	0.015	4.43 ± 0.61 ^{abcd}	0.14
2.25% Sorbitol	1.051 ± 0.058 ^{defg}	0.018	4.36 ± 0.55 ^{abc}	0.18
3.0% Sorbitol	0.593 ± 0.089 ^{ab}	0.019	3.67 ± 0.47 ^{a}	0.10
1.5% Sorbitol + 0.1% EDTA	1.178 ± 0.224 ^{efg}	0.051	4.25 ± 1.30 ^{abc}	0.30
1.5% Sorbitol + 0.15% Tween 80	1.232 ± 0.229 ^{fg}	0.052	4.68 ± 0.55 ^{abcd}	0.13
1.5% Sorbitol + 0.075% SP30	1.081 ± 0.142 ^{defg}	0.032	4.22 ± 0.59 ^{abc}	0.13
1.5% Sorbitol + 0.075% SP50	1.275 ± 0.197 ^{g}	0.045	4.30 ± 1.04 ^{abc}	0.24
1.5% Sorbitol + 0.075% S970	0.960 ± 0.188 ^{cdef}	0.046	3.71 ± 0.28 ^{a}	0.07

SD, standard deviation; SE, standard error; Means ($n \geq 15$) bearing different superscripts are significantly different ($P < 0.05$, Scheffé test).

4. Conclusions

This work demonstrated the effect of nisin, malic acid and natamycin-impregnated whey protein films against *L. monocytogenes*, *Ps. aeruginosa* , *Y. lipolytica* and *P. commune* and *P. chrysogenum*. The ability of the studied films to be a carrier of antimicrobials without significantly compromising the mechanical properties of films was demonstrated. Concerning the water vapour permeability, the films with glycerol at 3.0% showed the higher values and the films with sorbitol at 1.5% had the lower values. The overall results of antimicrobial assay, mechanical and WVP tests allow us to suggest the following formulation to be later evaluated in an experimental assay to wrap cheese: whey protein isolate (7.0%), malic acid (3.0%), sorbitol (1.5%), nisin (50 IU/ml), natamycin (0.002 g/ml). EDTA can also be used, since it increased the inhibitory power against *L. monocytogenes, P. commune* and *P. chrysogenum*. A concentration higher than 0.1% needs to be investigated to demonstrate inhibition against *Ps. aeruginosa*.

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CAPÍTULO VII

Considerações finais e perspectivas futuras

1. Considerações finais

Com a realização deste trabalho foi possível obter um conjunto de novas informações sobre a problemática ligada à contaminação por *Listeria monocytogenes* de queijos de ovelha feitos com leite cru e a existência de estirpes recorrentes em queijarias. Por outro lado, com o desenvolvimento de revestimentos edíveis e antimicrobianos, foi possível contribuir para o controlo do crescimento e sobrevivência de microrganismos naturalmente presentes na casca de queijo, cujo consumo constitui um perigo para a saúde pública.

Tendo em conta os objectivos previamente definidos, e referidos no Capítulo I, concluiu-se que os mesmos foram cumpridos. Com base nos resultados obtidos passaremos agora a referir as principais conclusões alcançadas:

- A análise molecular das 185 estirpes pertencentes às espécies *L. monocytogenes* (n=123), *L. innocua* (n=60) e *L. seeligeri* (n=2), isoladas entre 1995 e 2004 a partir de queijo de ovelha, de leite cru, de solução de cardo, de salmoura, de solução fungicida, de fezes e de zaragatoas a superfícies no interior das queijarias permitiu-nos concluir que a entrada de leite cru contaminado na queijaria, bem como a lavagem dos queijos e a imersão destes em salmoura, constituem pontos de contaminação por *L. monocytogenes.* Verificou-se ainda a persistência de alguns tipos moleculares na mesma queijaria em diferentes anos.

- O estudo de uma ovelha lactante com uma mamite sub-clínica (sem sinais visíveis de infecção), portadora assintomática de *L. monocytogenes*, permitiu-nos concluir que a contaminação era restrita ao lado esquerdo do úbero, o qual excretava a bactéria numa concentração média de 10^2 CFU/ml de leite, o qual apresentava um elevado número de células somáticas ($2{,}7 \times 10^6$ cells/ml). Através da discriminação dos isolados obtida por PFGE, o estudo da listeriose ovina referido acima demonstrou evidência epidemiológica de persistência de uma única estirpe de *L. monocytogenes* que ocasionou a mamite sub-clínica. Esta infecção foi a causa da contaminação do leite crú usado para produzir queijo não pasteurizado. Os mesmos tipos moleculares (AFLP IV-1, PFGE 11,

serogrupo molecular 4b) foram identificados no leite cru da ovelha com listeriose, nos queijos e na máquina de lavar os queijos.

- A avaliação da sensibilidade à nisina (nas concentrações de 5, 10, 50, 100 e 200 IU/ml) em meio sólido, de 219 estirpes de *L. monocytogenes* e *L. innocua* permitiu-nos concluir que houve um comportamento relativamente homogéneo das estirpes analisadas quanto ao efeito inibidor da nisina nas condições testadas (pH 5,5 e 20ºC, pH 5,5 e 37ºC, e pH 6,8 e 37ºC). A maioria das estirpes de *L. monocytogenes* apresentou valores para a Concentração Mínima Inibitória (CMI) de 50 ou 100 IU de nisina / ml. A maior tolerância à nisina foi verificada quando as estirpes foram incubadas a 37ºC no meio com pH 6,8, apresentando algumas delas, nestas condições, o desenvolvimento de resistência espontânea, com uma frequência média de $1{:}10^4$. A eficácia da nisina aumentou com a diminuição do pH, facto que estará na origem dos resultados mais inibidores apresentados no ensaio com o valor mais baixo de pH testado (5,5). Quando se estudou o efeito da nisina (50 IU/ml) ao longo do tempo em meio líquido (TSYGB) e em cultura estática, verificou-se que o efeito inibidor era temperatura-dependente a pH 6,8.

- A avaliação do potencial de virulência de 7 estirpes de *L. monocytogenes* previamente seleccionadas, incluindo a estirpe associada ao caso de listeriose ovina, através da realização de um teste de invasão celular *in vitro* usando células animais HT-29, permitiu-nos concluir que as estirpes analisadas eram virulentas quando comparadas com estirpes padrão, não tendo havido nenhuma estirpe testada identificada como não virulenta.

- Foi desenvolvida uma formulação para a preparação de revestimentos edíveis e antimicrobianos, com pH 3, com o objectivo de revestir os queijos e controlar a flora microbiana patogénica ou de alteração existente naturalmente na casca. Estes revestimentos foram elaborados com base em 7,0% de isolado proteico de soro de leite (WPI), 3,0% de glicerol e ácido málico (3,0%) e nisina (50 IU/ml). O ácido málico foi escolhido após um estudo comparativo com ácido cítico (1,5% e 3,0%), ácido fórmico (1,5% e 3,0%), ácido láctico (1,5% e

3,0%), ácido ascórbico (1,5% e 3,0%), ácido acético (1,5%) : ácido láctico (1,5%), ácido acético (1,5% e 3,0%), ácido fumárico (1,5% e 3,0%) e ácido málico (1,5%). Neste estudo foram avaliadas as propriedades antimicrobianas e mecânicas dos filmes (% de elongação e força máxima de ruptura) e as propriedades reológicas (viscosidade e viscoelasticidade) dos fluidos filmogénicos. Concluiu-se que nem todos os ácidos permitiam a formação de filmes intactos após secagem e que as propriedades avaliadas estavam fortemente dependentes do tipo e da concentração de ácido usado. Concluiu-se ainda que a utilização nestes revestimentos de um ácido orgânico era por si só inibidor do crescimento de *L. monocytogenes*; no entanto, verificou-se que a adição de nisina aos mesmos aumentava este efeito inibibor.

- Quando se passou à fase de optimização dos filmes produzidos, estudou-se o efeito da incorporação de ésteres de sacarose e de dois tipos de plastificantes (sorbitol e glicerol) na permeabilidade ao vapor de água, tendo-se concluído que os ésteres de sacarose não alteravam significativamente este parâmetro, o qual era dependente do tipo e da concentração (1,5%, 2,25%, 3,0%, p/v) do plastificante usado. A um aumento da concentração de plastificante correspondia um aumento dos valores de permeabilidade ao vapor de água. Para as mesmas concentrações de agente plastificante, os filmes produzidos com sorbitol apresentavam valores mais baixos.

- O estudo de outros agentes inibidores e/ou adjuvantes, com o objectivo de alargar o espectro da acção inibidora dos filmes a uma gama mais vasta de microrganismos, que incluísse também bactérias Gram-negativas e fungos, permitiu-nos concluir que a incorporação de ésteres de sacarose aumentava o efeito inibidor da nisina e do ácido málico sobre as bactérias da espécie *L. monocytogenes* mas diminuia significativamente o efeito inibidor da natamicina sobre os fungos (*Yarrowia lipolytica* e *Penicillium commune, P. chrysogenum* e *P. roquefortii*). Não obstante, em todos os casos verificou-se inibição do crescimento de todos os grupos de microrganismos estudados, não só por baixo do disco mas igualmente em redor deste. O EDTA e o Tween80

potenciaram o efeito inibidor da nisina e ácido málico no caso de *L. monocytogenes* mas não tiveram qualquer efeito no caso de *Ps. aeruginosa*.

2. Perspectivas futuras

Como resultado das conclusões anteriormente apresentadas, novos temas de estudo poderão ser no futuro abordados e aprofundados, nomeadamente:

- Avaliar a incorporação de outros agentes com características edíveis, que permitam reduzir significativamente a permeabilidade ao vapor de água destes filmes, já que a elevada higroscopicidade é uma característica dos filmes de natureza proteica em geral e que limita a sua utilização no revestimento de produtos sujeitos a atmosferas húmidas ou com elevadas humidades intrínsecas, como é o caso do queijo.

- Verificar a difusibilidade dos agentes antimicrobianos incorporados na matriz proteica dos filmes, já que é importante que esta seja limitada e se faça de forma lenta e gradual, exercendo assim uma acção mais eficaz e duradoura onde é necessário, ou seja, na casca.

- Testar outros agentes inibidores para o controlo da bactéria Gram-negativa *Pseudomonas aeruginosa*, ou testar os mesmos agentes mas a concentrações superiores às usadas no último ensaio, já que esta bactéria foi o microrganismo que se mostrou mais difícil de inibir.

- Proceder a um ensaio de revestimento de queijos artificialmente contaminados, usando para tal uma formulação previamente optimizada de fluidos filmogénicos, de forma a verificar a sua eficácia prática.

ANEXO I

Table 1. Strains of *Listeria* isolated from soft cheese

(Tabela inserida no artigo “Prevalence and characterization of *Listeria monocytogenes* isolated from soft cheeses” (Pintado *et al.* (2005). *Food Microbiology*. 22, 79-85)

Table 1. Strains of *Listeria* isolated from soft cheese.

Producer	Source of sample[a]	Cheese number	Enrichment[b]	Species	Seroype	Phage type	Code	ISALC strain no.
A	A	1	1	*L. monocytogenes*	4b	2389:2425:47:52:108:340	1	95.7
A	A	2	1,2,3	NG				
A	A	3	1	*L. monocytogenes*	1/2b	575	2	95.14
A	A	4	1	*L. monocytogenes*	1/2b	575	2	95.1
A	A	5	1,2,3	NG				
A	A	6	1	*L. monocytogenes*	4b	2389:2425:47:52:108:340	1	95.9
A	A	7	1,2,3	NG				
A	A	8	1	*L. monocytogenes*	1/2b	575	2	95.15
A	A	9		NG				
A	A	10	1,2,3	*L. monocytogenes*	ND	NT	ND	
A	A	11	1,3	*L. monocytogenes*	4b	2389:2425:3274:2671:47:108:340	3	95.3
			2	*L. monocytogenes*	4b	2389:3552:2425:3274:2671:47:52:108:340:312	4	95.2
A	A	12	1,2,3	NG				
A	A	13	1	*L. monocytogenes*	ND	NT	ND	
A	A	14	1,2,3	NG				
A	A	15	1	*L. monocytogenes*	4b	2389:3552:2425:47:52:108:340	5	95.13
			3	*L. monocytogenes*	4b	2389:2425:47:52:108:340	1	95.17
A	A	16	1	*L. monocytogenes*	4b	2389:2425:47:52:108:340	1	95.16
			2	*L. monocytogenes*	ND	NT	ND	
A	A	17	1	*L. monocytogenes*	ND	NT	ND	
A	A	18	1	*L. monocytogenes*	4b	2389:2425:47:52:108:340	1	95.8
A	A	19	1	*L. monocytogenes*	ND	NT	ND	
			1	*L. innocua*	ND	NT	ND	
A	A	20	1	*L. seeligeri*	ND	NT	ND	
			2,3	*L. monocytogenes*	ND	NT	ND	
A	A	21	1	*L. monocytogenes*	ND	NT	ND	
A	A	22	1	*L. monocytogenes*	4b	2389:2425:47:52:108:340	1	95.4
A	A	23	1,3	*L. monocytogenes*	ND	NT	ND	
A	A	24	1	*L. monocytogenes*	ND	NT	ND	
B	B	25	1,2,3	*L. innocua*	ND	16:4211:4286:1090:5337:4276:4277:1807	10	
B	B	26	1,2,3	NG				
B	B	27	1,2,3	NG				
B	B	28	1,2,3	NG				
B	B	29	1	*L. innocua*	ND	ND	ND	
			3	*L. innocua*	ND	16:4211:4286:1090:5337:1807	11	
C	C	30	1	*L. monocytogenes*	4b	3552:2425:52:108:340:312	8	95.19
			2,3	*L. monocytogenes*	4b	2671:108:312	7	95.5
C	N	31	1	*L. monocytogenes*	4b	3552:2425:52:108:340:312	8	95.6
			2,3	*L. monocytogenes*	4b	2671:108:312	7	95.23
C	N	32	1,3	*L. monocytogenes*	4b	2671:108:312	7	95.20
			2	*L. monocytogenes*	4b	3552:2425:52:108:340:312	8	95.24
D	D	33	1,3	*L. innocua*	ND	NT	ND	
D	D	34	1	*L. seeligeri*	ND	3665	ND	
			2,3	*L. innocua*	ND	NT	ND	
D	D	35	1,2,3	*L. innocua*	ND	NT	ND	
D	D	36	1	*L. monocytogenes*	ND	NT	ND	
			2	NG				
			3	*L. monocytogenes*	1/2a	1967:10:19:43:387	9	

E	N	37	1,2,3	*L. innocua*	ND	16:4211:4286:1090:5337:4276:4277:1807	10	
E	N	38	1	*L. innocua*	ND	16:4211:4286:1090:5337:4276:1807	12	
			2,3	*L. innocua*	ND	16:4211:4286:1090:5337:4276:4277:1807	10	
E	N	39	1	*L. innocua*	ND	16:4211:4286:1090:5337:4276:1807	12	
			2	*L. innocua*	ND	16:4211:4286:5337:4276:4277:1807	12	
			3	*L. innocua*	ND	ND	ND	
E	N	40	1,2,3	*L. innocua*	ND	16:4211:4286:1090:5337:4276:1807	12	
F	F	41	1,2,3	*L. monocytogenes*	4b	2389:3552:47:52:108:340:312	6	95.18
F	F	42	1,2,3	NG				
F	N	43	1,3	*L. innocua*	ND	16:4211:4286:1090:5337:4276:1807	12	
			2	*L. innocua*	ND	ND	ND	
G	N	44	1	*L. innocua*	ND	16:4211:4286:1090:5337:4276:4277:1807	10	
			3	*L. innocua*	ND	16:4211:4286:1090:5337:4276:1807	12	
G	N	45	1	*L. innocua*	ND	16:4211:4286:1090:5337:1807	11	
			2,3	*L. monocytogenes*	ND	NT	ND	
G	N	46	1,3	*L. monocytogenes*	ND	NT	ND	
H	H	47	1,2,3	NG				
H	N	48	1,2,3	NG				
H	N	49	1,2,3	NG				
I	N	50	1,2,3	*L. innocua*	ND	16:4211:4286:1090:5337:4276:4277:1807	10	
I	N	51	1	*L. innocua*	ND	16:4211:4286:1090:5337:4276:1807	12	
I	N	52	1,2,3	NG				
I	N	53	1,2,3	*L. monocytogenes*	4b	2671:108:312	7	95.21
J	N	54	1,2,3	*L. innocua*	ND	16:4211:4286:1090:5337:4276:4277:1807	10	
J	N	55	1	*L. innocua*	ND	16:4211:4286:1090:5337:4276:1807	12	
			2,3	*L. innocua*	ND	16:4211:4286:1090:5337:4276:4277:1807	10	
J	N	56	1,2	*L. innocua*	ND	16:4211:4286:1090:5337:4276:1807	12	
			3	*L. monocytogenes*	4b	2671:108:312	7	95.10
J	N	57	1	*L. innocua*	ND	16:4211:4286:1090:5337:4276:1807	12	
			3	*L. monocytogenes*	4b	2671:108:312	7	95.22
L	L	58	1	*L. innocua*	ND	NT		
			2,3	*L. monocytogenes*	4b	2671:108:312	7	95.12
L	N	59	1	*L. innocua*	ND	16:4211:4286:1090:5337:4276:1807	12	
M	M	60	1	*L. innocua*	ND	NT		
M	M	61	2	*L. innocua*	ND	NT		
M	M	62	1,2,3	NG				
M	M	63	1,2,3	NG				

[a] Source A was a co-operative that made, matured and sold cheese, using milk supplied by several farms. Source N was a co-operative that received, matured and sold cheese from producer farms B-M.

[b]Enrichment 1 = MFB+BLEB , Enrichment 2 = LEB-2 d, Enrichment 3= LEB-7 d, NT = Not Typeable, ND = Not Determined, NG = Negative.

ANEXO II

Publicações e comunicações

Publicações e comunicações

Grant, K.; Hampton, M.; Halford-Maw, R.; McLauchlin, J.; Pintado, C.M.B.S.; Threlfall, E.J. (2005). Molecular identification and characterization of *Listeria monocytogenes*. *Health Protection Agency Annual Conference 2005*, 12-14 September, Warwick University, London, United Kingdom.

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Pintado, C.M.B.S.; Ferreira, M.A.S.S.; Sousa, I. (2008). Properties of edible films whey-based as influenced by organic acids and antimicrobial agents. (p. 103-106). *In:* Guerrero, A.; Muñoz, J.; Franco, J. M. (Ed.). *Rheology in product design and engineering*. Grupo Español de Reologia - Real Sociedad Española de Química (GER/RSEQ). Madrid, Espanha. (Book of Proceedings Iberian Meeting on Rheology,IBEREO2008,ISBN:978-84-608-0779-7).

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Pintado, C.M.B.S.; Ferreira, M.A.S.S.(2009). High nisin susceptibility of *Listeria* spp. wild-type strains isolated from dairies with traditional cheese preservation in Portugal. *Letters in Applied Microbiology* (*submitted*).