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Hazards and Control of Risks in Artesanal Meat Products in Portugal

Thesis submitted to the *Universidade Católica Portuguesa*, to attain the
degree of PhD in Biotechnology with specialisation in Microbiology

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Maria Isabel Pereira da Silva Campelos

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“The known is finite, the unknown infinite; intellectually we stand on an islet in the midst of an illimitable ocean of inexplicability. Our business in every generation is to reclaim a little more land.” Thomas Henry Huxley. 1884

“Remember that all models are wrong; the practical question is how wrong do they have to be to not be useful”. George Edward Pelham Box**,** 1987

“The best model of a cat is another cat or, better, the cat itself”, Norbert Wiener, 1970

To my Father

GENERAL INTRODUCTION

"Salpicão de Vinhais" and "Chouriça de Vinhais" are traditional dry-fermented smoked meat sausages produced in the Vinhais, a small region of Trás-os-Montes. These sausages are registered as Protected Geographical Indication (PGI) and are a significant economic and cultural asset. The production of this kind of product is mainly empirical, developed and perfected through centuries. The scientific knowledge of this sausage variety is limited. Hence, the collection of sound, scientific knowledge regarding its microbiological and chemical characteristics is a necessity and a helpful tool for producers, retailers, official control agencies and consumers, providing for much needed information regarding quality and food safety.

Quantitative microbiological risk assessments aim at evaluating the risks, namely the probabilities and severities of adverse health effects for consumers, resulting from the exposure to pathogenic microorganisms present in foods, and implementing appropriate management options. Governments need risk assessment in order to select risk management measures for food trade at national and international levels, avoiding risks to the population. But also, risk assessment can be useful at production, at retailer, and at consumer levels.

This work aims to collect scientific data that could, partially, fill the gaps in knowledge regarding these products, analyse the data and develop a risk-based study, according to an internationally accepted framework and finally, to explore the effect of hypothetical risk management measures on the safety of consumers, regarding traditional dry fermented meat smoked sausages.

To perform a quantitative microbiological risk assessment, relevant data was either extracted from experimental data and when this was not possible, from relevant scientific literature. To perform the exposure assessment, a probabilistic model was developed. Since the physico-chemical (intrinsic) characteristics of the final product traditional dry fermented smoked sausages, fall near the boundaries that allow, or not, growth of *L. monocytogenes*, a growth/no growth equation, specific for the conditions of "Salpicão" and "Chouriça de Vinhais" was developed to be included in the model. Though official organisations like the European Food Safety Agency, state the relevance of growth/no growth boundaries, few risk assessments incorporate this in their exposure assessment models. Traditional dry fermented smoked sausages present low to medium risk to the health of consumers. The use of more restrictive Performance Objectives during processing and/or distribution will result in a further reduction of risk. A more rigorous control of final product water activity and the use of a bacteriocinogenic bioprotective culture during production may contribute significantly to reducing the risk of listeriosis in consumers of these Traditional Dry Fermented Smoked Sausages

ABSTRACT

"Salpicão de Vinhais" and "Chouriça de Vinhais" are traditional dry-fermented smoked meat sausages produced in Vinhais, a small region of Trás-os-Montes. The scientific knowledge of this sausage variety is limited. This work aims to collect scientific data that could, partially, fill the gaps in knowledge regarding these products, analyse the data and develop a risk-based study, according to an internationally accepted framework and finally, to explore the effect of hypothetical risk management measures on the safety of consumers, regarding traditional dry fermented meat smoked sausages.

Seventy seven samples of "Salpicão" and "Chouriça de Vinhais" were purchased from producers, local markets and retail stores. Their microbiological and physical chemical characteristics were analyzed. The same analyses were performed on the products during the production processes. The raw materials and ingredients were also analyzed. Regarding the pathogenic flora, *Staphylococcus aureus*, spores of sulphite-reducing clostridia, *Escherichia coli* 0157:H7, *Yersinia* spp. and *Salmonella* spp., were not detected in any of the samples analyzed; *Listeria monocytogenes* was detected in 14.3% of the samples. The manufacturing process, namely fermentation, ripening/drying and smoking reduced the numbers of pathogen and hygiene indicator microorganisms. According to the Commission Regulation (EC) 2073/2005, 39% of the contaminated samples of "Salpicão" and "Chouriça de Vinhais" were able to support growth of *L. monocytogenes*. A quantitative microbiological risk assessment using a probabilistic model was developed. Considering the Portuguese population, with data obtained in this

work, the calculated risk of listeriosis, for the intermediate age sub-population was 0.1297 cases per year, for the perinatal sub-population, 1.3695 cases per year, and elderly sub-population 0.1995 cases per year. This means that Traditional Dry Fermented Smoked Sausages constitute a low risk for the Intermediate Age and Elderly population (less than 1 case per annum) and an intermediate risk for the Perinatal populations, considering this last group of consumers as adult pregnant women (1-10 cases per annum).

The effect of several putative risk management actions such as the adoption of a Performance Objective of 10 CFU/g, 1 CFU/g and 0.04 CFU/g reduced the risk of listeriosis for all sub-groups, at the end of shelf life, by 25.5%, 58.4% and 58.6%, respectively.

The effect of bacteriocinogenic strain of *Pediococcus acidilactici* HA6111-2, previously isolated from "alheira", was studied on both "Salpicão" and "Chouriça", in a challenge test against a cocktail of *Listeria innocua*, at pilot scale. A reduction of the numbers of *Listeria innocua* was achieved in all batches, being the biphasic equation the model that gave the better fit. The use of a bacteriocinogenic strain added to the batter reduced the risk of listeriosis for all sub-groups by 26.7%; obtaining a final product with a_w below 0.92, supposedly below the growth limits of *L. monocytogenes*, reduces the risk by 8.6% in all sub-groups of population; the combination of the bacteriocinogenic strain and the limit of a_w 0.92 reduces the risk by 41.0%. Traditional dry fermented smoked sausages present low to medium risk to the health of consumers. The use of more restrictive Performance Objectives during processing and/or distribution will result in a further reduction of risk.

A more rigorous control of final product water activity and the use of a bacteriocinogenic bioprotective culture during production may contribute significantly to reducing the risk of listeriosis in consumers of these Traditional Dry Fermented Smoked Sausages (TDFSS).

RESUMO

O Salpicão de Vinhais e a Chouriça de Vinhais são enchidos fermentados, secos e fumados, produzidos em Vinhais, uma pequena região de Trás-os-Montes.

Este trabalho pretende recolher dados científicos que possam preencher, ainda que parcialmente, os lapsos no conhecimento deste tipo de produtos, de acordo com uma abordagem aceite internacionalmente e, finalmente, explorar o efeito potencial que as medidas de gestão de risco podem ter na segurança dos consumidores, relativamente a enchidos tradicionais, fermentados, secos e fumados.

Setenta e sete amostras de "Salpicão" e "Chouriça de Vinhais" foram compradas em produtores, mercados locais e hipermercados. As suas características microbiológicas e físico-químicas foram analizadas. As mesmas análises foram realizadas ao produto em curso, em diferentes fases do processo produtivo. As matérias-primas e ingredientes também foram analisadas. Relativamente à flora patogénica, não foram detectados esporos de *Staphylococcus aureus*, esporos do género Clostridia sulfito-redutores, *Escherichia. coli* 0157:H7, *Yersinia* spp. e *Salmonella* spp; *Listeria monocytogenes* foi detectada em 14.3% das amostras. O processo de fabrico, nomeadamente fermentação, maturação/secagem e fumagem reduziram o número de patogénicos e microrganismos indicadores de higiene. De acordo com o Regulamento da Comissão (CE) 2073/2005, 39% das amostras de "Salpicão" e "Chouriça de Vinhais" contaminadas eram capazes de permitir o crescimento de *L. monocytogenes.* Foi desenvolvida uma avaliação quantitativa de risco microbiológico. Considerando a população portuguesa, com os dados obtidos neste trabalho, o risco calculado de listeriose seria de 0.1297 casos por

ano para a população de idade intermédia, 1.3695 casos por ano para a sub-população perinatal, e 0.1995 casos por ano para a sub-população sénior. Isto significa que Enchidos Tradicionais Secos, Fermentados e Fumados constituem um alimento de baixo risco para para a população de idade intermédia e população sénior (menos de 1 caso por ano) e são um alimento de risco intermédio para a população perinatal, ou seja, mulheres grávidas adultas (entre 1 a 10 casos por ano).

A adopção de um Objectivo de "Performance" de atingir 10 UFC/g, 1 UFC/g e 0,04 UFC/g no produto final reduziram o risco de listeriose para todos os sub-grupos da população, em 25.5%, 58.4% e 58.6%, respectivamente.

O efeito da estirpe bacteriocinogénica de *Pediococcus acidilactici* HA6111-2, previamente isolada em alheira, foi estudada tanto no Salpicão como na Chourila de Vinhais, num "challence test" contra um cocktail de *Listeria innocua*, à escala piloto. Foi observado, em todos os lotes, uma redução dos números de *Listeria innocua*, abaixo do limite de detecção (1.5 log UFC/g), A equação bifásica foi que forneceu o melhor ajuste da redução dos números de *Listeria innocua, devido ao* da estirpe bacteriocinogénica de *Pediococcus acidilactici* HA6111-2. A aplicação de uma estirpe bacteriocinogénica à adoba reduziu o risco de listeriose, em todos os sub-grupos populacionais em 26.7%; obter um produto final com a_w inferior a 0.92 reduziu o risco de listeriose, em todos os sub-grupos populacionais em 8.6%; a combinação destes dois factores reduziu o risco de listeriose, em todos os sub-grupos populacionais em 41.0%.

Os Enchidos Tradicionais Secos, Fermentados e Fumados apresentam um risco baixo ou médio para a saúde dos consumidores. A aplicação de Objectivos de

"Performance" durante o processamento e/ou distribuição resulta numa redução do risco. Um controlo mais rigoroso da actividade de água do produto final e o uso de uma cultura bioprotectiva bacteriocinogenica durante a produção, podem contribuir para reduzir significativamente o risco de listeriose nos consumidores deste tipo de produto tradicional.

KEYWORDS

"Chouriça"

"Salpicão"

Bioprotective cultures

Dry fermented meat smoked sausage

Lactic acid bacteria

Listeria monocytogenes

Listeria spp.

Quantitative microbiological risk assessment

Monte Carlo

Probabilistic simulation

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LIST OF ABBREVIATIONS

μ	Population mean
A	Difference between the initial and final population (N_0 - N_f)
A_f	Accuracy factor
AFNOR	Association Française de Normalisation
ALOP	Appropriate level of protection
ANOVA	Analysis of variance
APC	Aerobic plate count
At	Adjustment function
ATCC	American Type Culture Collection
a_w	Water activity
BA	Biogenic Amines
bac	Bacteriocinogenic
BHI	Brain Heart Infusion
BSE	Bovine Spongiform Encephalopathy
CAC	Codex Alimentarius Comission
CDC	Centers for Disease Control and Prevention
CFSAN	Center for Safety and Applied Nutrition
CFU	Colony-forming unit
CIN	Cefsulodin, irgasan and novobiocin
CNC	Coagulase-Negative Cocci
CP	Consumption period

D	Dose
D	time, in days, at a given temperature, required to destroy 1 log cycle (90%) of the target microorganism
DF/*df*	Degree of freedom
D-LDH	D - lactate dehydrogenase
DNA	Deoxyribonucleic Acid
E.	*Escherichia*
EC	European Community
Ec.	*Enterococcus*
ECDC	European Centre for Disease Prevention and Control
EDTA	*Ethylenediamine Tetraacetic Acid*
EFSA	European Food Safety Authority
EHEC	Enterohemorrhagic
ESB	Escola Superior de Biotecnologia
ETG	Traditional Speciality Guaranteed
EU	European Union
F	*F* ratio
f	fraction of initial population in the major population
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service
FSO	Food Safety Objectives
GPI	Protected Geographical Indication

GRAS	*Generally Regarded As Safe*
GT	Generation time
GTP	Glutamate-pyruvate transaminase enzyme
HACCP	Hazard Analysis and Critical Control Points
HHP	High hydrostatic pressure
HUS	Hemolytic uremic syndrome
ID50	Median infective dose
IDRHA	Instituto de Desenvolvimento Rural e Hidráulica
INE	Instituto Nacional de Estatística
ISO	International Organization for Standardization
ITC	Irgasan, ticarcillin and potassium chlorate
k	Inactivation rate
k_{max}	Specific inactivation rates
L.	*Listeria*
L.m	*Listeria monocytogenes*
LAB	Lactic Acid Bacteria
LABpos	inoculated with *Ped. acidilactici* HA-6111-2 (bacteriocinogenic strain
Lact.	*Lactococcus*
Lb.	*Lactobacillus*
LD50	Median lethal dose
LDH	lactate dehydrogenase
LI	inoculated with a cocktail of *L. innocua*

LI+LABneg	inoculated with cocktail of *L. innocua* and *Ped. acidilactici* HA-2485-3 (non bacteriocinogenic strain)
LI+LABpos	inoculated with cocktail of *L. innocua* and *Ped. acidilactici* HA-6111-2 (bacteriocinogenic strain)
L-LDH	L - lactate dehydrogenase
LOF	lack of fit
Logit (P)	ln[P/ (1−P)]
m	Sample mean
MADRP	Ministério da Agricultura, Desenvolvimento Rural e Pescas
M-H	Muller-Hinton
MIC	Minimum Inhibitory Concentration
MKTT	Mueller-Kauffmann Tetrathionate-Novobiocin Broth
MPN	Most Probable Number
MRA	Microbial risk analysis
MRS	de Man-Rogosa-Sharpe
MSE	Mean Sum of Squared Errors
N_0	Initial contamination
na	Not available
NADH	Nicotinamide adenine dinucleotide hydride
N_b	Batter contamination
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Collection of Type Cultures
nd	Not detected

N_i	Initial contamination in new estimate
N_{max}	Maximum contamination
$N_{predicted}$	Predicted contamination
NRC	National Research Council
N_t	Contamination at time t
NZFSA	New Zealand Food Safety Authority
OD	Optical Density
ODc	Optical Density cut-off
OIE	Office international des épizooties
P	Probability
PALCAM	PolymyxinAcriflavin-LiCl-Ceftazidime-Aesculin-Mannitol
PC	Performance Criteria
PCR	Polymerase Chain Reaction
PDO	Protected designation of origin
Ped.	*Pediococcus*
PGI	Protected Geographical Indication
PHLS	Public Health Laboratory Service
PO	Performance Objectives
PSB	Peptone, sorbitol and bile
QMRA	Quantitative Microbiological Risk Assessment
R	Model parameter specific
R^2	coefficient of determination
RA	Risk Analysis

RAPD	Random Amplified Polymorphism DNA
RPF	Rabbit plasma fibrinogen supplement
RTE	Ready-to-Eat
RVS	Rappaport-Vassiliadis Soy Peptone Broth
s	Sample standard deviation
Salm.	*Salmonella*
SD	Standard Deviation
SDFS	Semi-dry fermented sausages
SDS-PAGE	Sodium Dodecyl Sulphate- PolyAcrylamide-Ge*l*-
SPS	Sanitary and Phytosanitary
SSDC	Sodium desoxycholate and calcium chloride
SSE	sum of Squared Errors
SSTO	sum of the squared differences
St.	*Staphylococcus*
Strep.	*Streptococcus*
T	Temperature
t_{4D}	Time for four decimal reduction
TAE	Tris–Acetate-EDTA
TBX	Tryptone, bile salt, X-GLUC agar
TDFSS	Traditional dry fermented, smoked sausage
TE	Tris-EDTA
t_s	shoulder period
TSA	Tryptic Soy agar

TSB-YE	Tryptic Soya Broth with Yeast Extract
TSBYE	Tryptic Soy Broth Yeast Extract
TSE	Transmissible Spongiform Encephalopathy
U	Unit
U.S.A	United States of America
UCP	Universidade Católica Portuguesa
UK	United Kingdom
USDA	United States Department of Agriculture
VTEC	Verotoxigenic
WHO	World and Health Organization
WTO	World Trade Organization
Y.	*Yersinia*
σ	Population standard deviation

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CHAPTER 1.INTRODUCTION

1.1 Dry fermented meat sausages

When humans began to gather and hunt food in excessive amounts, they must have experienced that, sooner or later, food became inedible, either because of its disagreeable taste or odour or because it caused illness. They must also have noticed that, drying food in the sun or above fire in caves or in tents, where smoking contributed additionally to taste and length of storage, food products were preserved for a longer time and with good palatability (Incze, 2007). For centuries, the transformation of pork meat into sausages has been a way to assure the supply of preserved meat all year around, in times when mechanical refrigeration was unknown. Fermentation and drying of meat products are probably the most ancient ways of preservation.

i) The meat processing sector

Traditional dry fermented sausage production has increased overall in Europe since the 1980's (Chevallier *et al.*, 2006). Dry fermented sausages are important meat products, and 4,967,000 tons of raw dry sausages were produced in the EU in 2006 (Eurostat, 2008). In Portugal, the consumption of meat was 100.5 kg/inhabitant/year, pork being the meat of major importance (42.5%) (MADRP, 2007). Protected designation of traditional products was valued, in 2007, as 70 million euro (MADRP, 2007).

ii) Classification of fermented sausages

All European countries have cultural traditions linked to food. However, probably due to the specific climatic conditions, there is a wider variety of production in the Southern European countries where, consequently, traditional products have a greater significance and economic importance (Jordana,, 2000). There is a wide variety of dry fermented products on the European market as a consequence of variations in the raw materials, formulations and manufacturing processes, originating from the habits and customs of the different countries and regions. In many European countries, the demand for traditional food products has increased (Talon *et al.*, 2008).

Mediterranean countries have a long history of producing these foods, often at local or regional level. These products are manufactured without the addition of starter cultures in small-scale processing units. Thus fermentation in traditional dry sausages relies on indigenous, non-controlled bacterial activity (Hugas *et al*., 2003; Ammor *et al.*; 2005; Lebert *et al.*,2007b; Talon *et al.*, 2008).

Fermented sausages can be divided into two groups: dry sausages that are ripened over 4 weeks (slightly acidic with a firm texture) or semi-dry sausages which are ripened for between 7 and 28 days and are less intensively dried (delivering a strongly acidic and a softer texture). Semi-dry fermented sausages have become very popular in Northern-Europe and the United States in the past 30 years (Houben and van‘t Hooft., 2005; Talon *et al.*, 2007).

Lücke (1998), proposed a classification of fermented sausages based on their curing time, final water activity ($a_{w)}$, and smoking process as described in Table 1.1.

Also, differentiations can be made on the basis of pH, with fermented sausages produced in Northern countries showing a pH between 4.8 and 5.1. Fermentation occurs rapidly at high temperatures, (traditional fermentation 18-24 °C; fast fermentation 22-26 °C; very fast American-style fermentation 32-43 °C) (Schwing and Neidhardt., 2007) resulting in a rapid fall in pH. Sausages produced in the Mediterranean countries present a higher pH of 5.3 to 6.2 (Klingberg *et al.*, 2005; Talon *et al.*, 2007; Danilovic *et al.*, 2011).

Table 1.1 Classification of fermented sausages

Category	Ripening times	Final water activity	Application of smoke
Dry, mould fermented	> 4 weeks	<0.90	No
Dry, mould fermented	> 4 weeks	<0.90	Yes (during fermentation)
Dry, no mould growth	> 4 weeks	<0.90	Yes or no
Semi-dry, mould fermented	< 4 weeks	0.90-0.95	No
Semi-dry, no mould growth	< 4 weeks (usually 10-20 days)	0.90-0.95	Yes (with exceptions)
Non-dried, spreadable	< 2 weeks	0.94-0.96	Yes or no

(adapted from Lücke, 1998).

Although the industrial production may require the use of starter cultures and control of the manufacturing process, artisanal products are increasingly

appreciated because of their sensory properties and authenticity (Samelis, 1999; Leroy et *al.,* 2006; Talon *et al*. 2007; Danilovic *et al.,* 2011).

The production of Mediterranean dry-fermented sausages is based, as in other varieties from Northern and Central Europe, on a combination of fermentation and drying, but in Mediterranean countries, air-drying, the addition of spices and longer ripening period are prevalent, together with the smoking process, though the latter is not generally applied (Ordóñez and Hoz., 2007).

1.1.1"Salpicão de Vinhais" and "Chouriça de Vinhais" – artisanal meat products

"Salpicão de Vinhais" and "Chouriça de Vinhais" are traditional dry-fermented meat sausages produced in Vinhais, a small region of Trás-os-Montes, a mountainous region in the Northeast of Portugal. This is an isolated and unfavoured region. As a consequence, the food production system of numerous indigenous agricultural families has been for familial-consumption. In this context, the transformation of pork meat is an ancestral practice that has created a great diversity of traditional meat products, with unique technological and sensory characteristics (Patarata *et al.*, 2004, 2008). The essential ingredient of "Salpicão de Vinhais" and "Chouriça de Vinhais" is raw pork meat from "bísaro", an autochthonous pig breed. The "bísaro" pork is one of the three autochthonous Portuguese breeds. These sausages are registered as Protected Geographical Indication (PGI). This means that they possess a specific quality, reputation or other characteristics attributable to that geographical origin, and the production

and/or processing and/or preparation take place in the defined geographical area, in this case, Vinhais (EU, 2006). The scientific knowledge about this sausage variety and the existing information in the scientific literature is limited (Ferreira *et al*, 2007a, 2009). The physicochemical characteristics of ‘‘Salpicão de Vinhais’’ and ‘‘Chouriça de Vinhais’’ are shown in Tables 1.2. and 1.3. The quality of these sausages is very variable, as there is very little uniformity between products manufactured by different small processors and meat industries. Actually, there does not exist an Official Standard of Quality which reflects the compositional characteristics which this type of sausage should possess, and to which the product made by the different manufacturers should conform.

Table 1.2. Minimum, maximum, mean and standard deviations of physico-chemical parameters and composition of ‘‘Salpicão de Vinhais’’ and ‘‘Chouriça de Vinhais’’

	Salpicão				Chouriça			
	Min.	Max.	Mean	SD	Min.	Max.	Mean	SD
pH	5.0	5.9	5.3	0.2	5.1	5.6	5.4	0.1
% NaCl	1.5	4.3	2.4	0.8	1.3	2.9	2.3	0.5
% Moisture	30.1	57.3	41.7	8.9	13.9	44.0	29.8	9.4
% Fat	3.6	26.4	16.2	8.0	20.4	68.2	36.1	14.3
% Total protein	31.6	43.5	35.3	4.0	12.3	35.9	27.2	6.7
% carbohydrate	<0.1	16.4	3.2	5.3	<0.1	21.3	3.7	6.7
Energy (kcal/100g)	176.0	374.0	299.7	70.4	329.0	497.0	412.0	68.0

Adapted from Ferreira *et al.* (2007a)

Table 1.3 Biogenic amine content in ‘‘Chouriça de Vinhais’’ and ‘‘Salpicão de Vinhais’’ (mg/kg)

Product	Histamine	Methylamine	Ethylamine	Tyramine	2-Phenyl ethylamine	Putrescine	Isoamylamine	Cadaverine
‘‘Salpicão’	0.2	0.0	0.0	13.4	0.5	0.4	0.0	0.1
‘Chouriça’	1.6	0.6	0.6	75.9	0.0	11.3	0.0	1.7

Adapted from Ferreira *et al.* (2007a)

There are two slaughterhouses and three transformation units, identified by official control agencies, that produce certified “Salpicão de Vinhais” and “Chouriça de Vinhais”, besides the home-made products, representing 5.86% of the national traditional sausage production. These industries produced 2810 kg of “Salpicão” at 40.00€/kg and 3096 kg of “Chouriça” at 30.00€/kg, in 2007. These are high-value products (for instance “alheira de Vinhais” PGI costs 8.00€/kg) and have the highest unit price among registered traditional meat products, in Portugal. Regarding their distribution, Fig. 1.1 quantifies the points of sale for these products. Despite the globalisation and standardization of food products, local resources represented by traditional products, although produced on a small scale, have a great economic impact and, consequently, contribute to a sustainable rural development (Vaz Velho *et al*., 2003; Ferreira *et al.*, 2006).

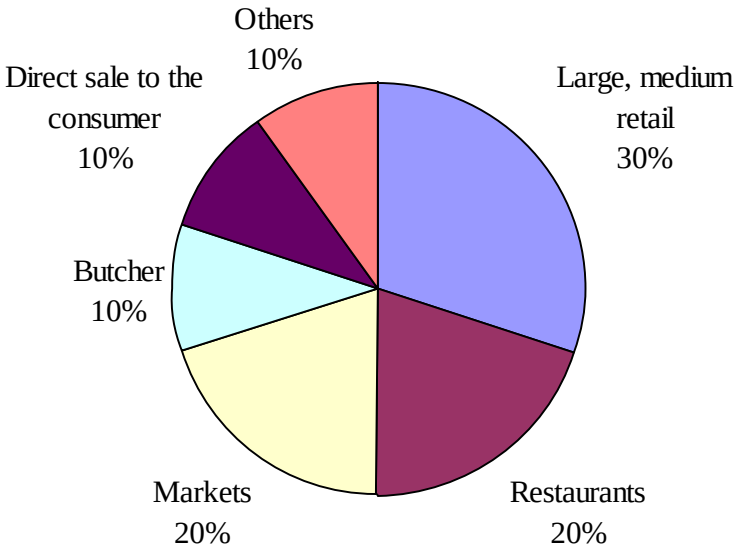


Fig.1.1 Points of sale of "Salpicão de Vinhais" and "Chouriça de Vinhais" (MADRP, 2007).

1.1.2 Dry fermented sausage manufacturing process

These sausages can be defined as the mixture of "bísaro" raw pork, pork fat drippings, salt, spices and typical wine, which undergo a natural drying-ripening process.

i) The meat is cut in large pieces, in the manufacture of "Salpicão" or minced, in the case of "Chouriça".

ii) The "adoba", a batter with red wine, salt, laurel, paprika, fresh or powdered garlic is prepared, to which the meat is added. The mixture is stored at 4 ºC, for 48 h, and although at a low temperature, the fermentation begins.

iii) The "adoba" is stuffed, manually or mechanically, into pork casings to form cylindrical formed sausages (ca. 15-20 cm long), in the case of "Salpicão", and horseshoe-shaped sausages (ca. 30-35 cm long) for "Chouriça";

iv) The fresh sausages undergo prolonged smoking processes. This process takes 4-5 weeks, for "Salpicão", and 3-4 weeks for "Chouriça". During smoking, fermentation, drying and ripening occur. The fermentation and ripening/drying do not always constitute two separate steps (Talon *et al.*, 2007).

A study in five producer units indicated that traditional smoking occurred between 4 and 15 ºC and relative humidity between 60 and 90% (Patarata *et al.*, 2004). Smoking temperatures, in traditional manufacture, are not controlled, varying according to the weather (Talon *et al.*, 2007). Traditionally, dry fermented smoked sausages should only be produced during the very cold winter months, but increasing economic interest has led to its production all year long, with some producers lacking appropriate infra-structures to control temperature, while others, more industrialised, do have refrigerated installations. Normally these products are consumed without further cooking, but they can be cooked (roasted or grilled) if the drying process is incomplete. Usually, these more perishable products are purchased by the consumer, directly from the producer, with this type of consumption in mind. Packaged and distributed sausages are expected to be fully stabilized, by a thorough smoking (drying) process. Because of its empirical origin, this process does not distinguish between fermentation, drying or smoking stages.

The fermentation step in the sausage production includes the period, in the sausage process, where pH decreases from approximately 5.7 to its lowest value, which could vary from 5.5 (low temperature fermented sausages) to 4.6 (or even 4.2 in high-temperature fermented sausages) depending on the sausage type

(Petäjä-Kannine and Puolanne, 2007). The fermentation lasts from less than 12 h to several days depending on the sausage style. Sausages fermented at high temperatures (37 °C or higher) quickly reach the lowest pH value, but temperatures of around 24 °C result in a pH of 4.6 to 5.0; lower temperatures usually result in a higher pH at a slower rate (Petäjä-Kannine and Puolanne, 2007*).* The variation in temperature and duration of the drying step leads to variable water content of traditional sausages at the end of drying ranging from 0.83 to 0.93 in French, Spanish, Portuguese and Italian sausages (Leroy *et al.*, 2009). One factor that stops fermentation is low pH, but the decrease of a_w will also inhibit further fermentation. The decrease of pH to values near the isoelectric point of proteins reduces their water retention capacity, facilitating posterior drying. Proteolysis (mainly due to muscular proteases, such as cathepsin D) and lipolysis (due to muscular and microbial lipases) start during fermentation producing free fatty acids, glycerol, monoglycerides, and diglycerides. (Petäjä-Kannine *et al.*, 2007). During drying-ripening, dehydration, continuing proteolysis and lipolysis occur, forming the compounds that contribute to obtaining the desired fermented sausage flavour profile (Di Cagno *et al.*, 2008; Patarata *et al.*, 2008). During the drying process, three zones can be distinguished corresponding to three typical steps occurring in a drying process: induction period, constant rate period, and falling rate period of drying. The induction period corresponds to the beginning of the drying process during which a heat transfer occurs from the air to the product, increasing the surface temperature up to the wet-bulb temperature (Andrés *et al*, 2007). When the surface reaches the wet-bulb temperature the total

amount of heat from the air is used to evaporate water, and because the rate of evaporation is lower than the rate of water transport to the surface, the drying rate is constant (constant rate period). As the drying process proceeds, the product dries and the water transport rate toward the surface decreases. The final step is the falling rate period or diffusion period of drying, when the removal of this water is done by diffusion. Internal moisture is more difficult to remove because it is more tightly bound and is protected by the insulating effect of the already dried material close to the surface (Andrés *et al.*, 2007). In some cases, the drying process also allows some biochemical reactions to develop taste and flavour, and it is known as a curing process. Smoke curing is a typical combined treatment, based on the combined action of enzymes and heat, which promote protein and lipid changes in the previously salted raw material. The treatment affects the sensorial quality, safety, and shelf life of the product, but the extent of these changes will depend on many factors, such as the type of smoking; the relative humidity, air velocity, temperature, density and composition of the smoke; and the time of smoking (Yean *et al*., 1998). Hajmeer *et al.* (2011) found that **s**moking was found to be effective against all of the five tested pathogens *(Campylobacter jejuni, Escherichia coli O157:H7, Listeria monocytogenes, Salmonella enterica* serotype Newport, and *Yersinia enterocolitica*), presumably because of the high temperatures (higher than 60ºC) employed.

Traditional smoking is divided into two categories:

- *Cold smoking*, with smoke temperatures between 20–25 °C and relative humidity of 70–80%. The duration of this process can be days or weeks because the smoke used is poor in aromatic and preserving components.

- *Hot smoking*, usually applied to meat products previously cooked or blanched. Temperatures can be about 75–80 °C, and the relative humidity must be high to avoid dehydration. With this procedure, the smoking time is reduced to hours (Yean *et al*. 1998; Andrés *et al*., 2007)

1.1.3 Microbiology of dry fermented meat sausages

Traditional dry sausage fermentation relies on natural contamination of raw materials by environmental flora. Each processing facility has a specific house flora, composed of useful microorganisms for the fermentation and flavour of sausage, as well as of spoilage and pathogenic flora (Chevallier *et al.*, 2006; Benito *et al.*, 2007). During the manufacture of dry-fermented sausages chemical and physico-chemical modifications occur, especially dehydration, fermentation of carbohydrates, acidification, development of a typical colour, lipolysis and oxidation of lipids, and proteolysis due to the activity of the different microbial groups (Comi *et al.*, 2005;Foulquié Moreno *et al.*, 2006; Garcia-Fontan *et al.*, 2007; Di Cagno *et al.*, 2008). Several investigations have established two groups of microorganisms as being mainly responsible for the transformations involved during fermentation and ripening of sausages. Lactic acid bacteria (LAB), in particular *Lactobacillus* spp., and Gram-positive coagulase negative cocci (CNC),

specifically *Staphylococcus* and *Kocuria* spp., are considered technologically fundamental (Talon *et al.*, 2007; Di Cagno *et al.*, 2008). Total LAB constitute the major microbiota of the traditional sausages. Lactic acid bacteria usually increase during the very first days of fermentation and they remain constant during ripening at 7 - 9 log CFU/g (Comi *et al.*, 2005; Talon *et al.*, 2007) or they can increase throughout the process and reach a similar final value (Lebert *et al.*, 2007b). Lactic acid bacteria growth is often correlated with the decrease in pH in the first stage of maturation (Petäjä-Kanninen and Puolanne., 2007). Enteroccocci generally have an initial level between 2 and 4 log CFU/g. Enterococci usually grow early during fermentation and remain constant at a level of 4 - 6 log CFU/g until the end of the whole process (Comi *et al.*, 2005; Drosinos *et al.*, 2005; Talon *et al.*, 2007). Enterococci are poor acidifiers and in traditional sausages of high pH they find good conditions for survival and growth (Hugas *et al*., 2003). Coagulase negative cocci constituted the second largest fraction of the microbiota, with a population of 4 - 6 log CFU/g (population, generally inferior to that of the LAB). Coagulase negative cocci sometimes grow during the fermentation period or they can grow during ripening (Comi *et al*., 2005) or throughout the process (Lebert *et al.*, 2007a). The numbers of spoilage bacteria, such as *Pseudomonas* present very different levels according to the source, and much of the time remaining stable or diminishing during fermentation and, during drying-ripening (Drosinos *et al.*, 2005; Chevallier *et al.*, 2006), although some authors have reported an increase during ripening (Comi *et al.*, 2005). The presence of foodborne pathogens in fermented meat sausages such as *Listeria monocytogenes*, *Escherichia coli*

O157:H7, *Salmonella spp*., *Clostridium botulinum*, *Staphylococcus aureus, Bacillus cereus* and *Yersinia enterocolitica* has been reported by several authors (Lahti *et al*., 2001; Thévenot *et al*., 2005a,b; Esteves *et al.*, 2006; Ferreira *et al*., 2007a,b; Colak *et al*, 2007; Esteves *et al.*, 2007; Cabedo *et al.*, 2008; Karakolev., 2009; Meloni *et al.*, 2009).

1.1.3.1 Lactic acid bacteria

Lactic acid bacteria are present in all fermented foods, have a long history of safe use and form part of the gut microflora of humans and animals. In addition, LAB produce a range of antimicrobial substances such as organic acids and bacteriocins that have been already exploited by the food industry (Maragkoudakis *et al*, 2009). Lactic acid bacteria are recognized to play an important role in food fermentations and food preservation through acidification (Talon *et al*, 2007*).* Several species of Lactobacillus (Lebert *et al*, 2007a) have been identified in traditional fermented sausages.

Lactic acid bacteria also produce small amounts of acetic acid, propionic acid, ethanol, acetoin, carbon dioxide, and pyruvic acid during fermentation, depending on the starter applied (if used), the carbohydrate substrate, and the sources of meat proteins and additives (Hugas *et al*., 2003).

Hydrogen peroxide is also produced by a large number of LAB lacking the enzyme catalase, in particular by *Lactobacillus* spp., that inhibits other microorganisms such as some *L. monocytogenes* strains, *St. aureus* and *Pseudomonas* spp. Some LAB strains are also able to produce antimicrobial

peptide compounds (bacteriocins), thereby enhancing the safety of fermented sausages (Albano *et al.*, 2007a,b; Talon *et al.*, 2008; Porto-Fett, 2010); many bacteriocins have the ability to inhibit the growth of pathogenic bacteria, such as *L. monocytogenes*.

1.1.3.2 Pathogenic bacteria

Consumers consider traditional fermented sausages safe foods. The simultaneous reduction in the a_w and pH partially inhibits the development of pathogenic bacteria during the various stages of sausage manufacture. However, they may still be present in the final product, causing a concern for the producers and for those responsible for public health, and have been the topic of study of several research groups. Outbreaks of foodborne illness associated with these products have occurred (Moore *et al.*, 2004; Lindqvist *et al.*, 2009). Different surveys have revealed the presence of *L. monocytogenes*, *E. coli*, *Clostridium* spp., and *St. aureus* not only in the final products, e.g., sausages, but also in different production levels. This can be due either to frequently contaminated raw materials, where there is a probability that some of the pathogenic organisms could cross the antimicrobial barriers imposed during processing, or to cross-contamination at any stage, including at retail. In both cases, pathogens may be present in the final product.

Salmonella spp. and *Yersinia enterocolitica*

Salmonella spp. and *Yersinia enterocolitica* b*oth blong to the genus Enterobacteriacea. Salmonella* has long been recognized as one of the most

important zoonotic pathogens of economic significance in animals and humans. Human salmonellosis is usually characterized by acute onset of fever, abdominal pain, nausea, diarrhoea and sometimes vomiting. Symptoms are usually mild and most infections are self-limiting, lasting a few days. However, in some patients, the infection may be more serious and the associated dehydration can become life threatening. In these cases, as well as when *Salmonella* causes bloodstream infection, effective antimicrobials are essential for treatment (Skandamis *et al.*, 2007a). There are numerous foodborne sources of *Salmonella*, including a wide range of domestic and wild animals and a variety of foodstuffs (meat and meat products, including sausages). *Salmonella* serotypes are well-known pathogens that have been implicated in a large number of outbreaks of foodborne disease in Europe (Cabedo *et al.*, 2008). *Salmonella* spp. may contaminate these products via contaminated raw meat, ingredients and/or processing equipment, and/or from post-processing contamination. These pathogens have been detected in raw meat and have also been shown to survive during certain sausage manufacturing processes (Colak *et al.*, 2007).

Yersinia spp., are Gram-negative bacilli, or coccobacilli. *Y. enterocolitica*, and, less commonly, *Y. pseudotuberculosis*, belong to a genus associated with diarrhoea in humans. *Y. enterocolitica* was found in low numbers in "alheiras", a traditionally fermented meat product, produced in the Northeast of Portugal (Esteves *et al.*, 2008).

During properly controlled sausage fermentation, *Enterobacteriaceae* counts slightly increased during fermentation (Comi *et al*, 2005; Fernandez-Lopez *et al*,

2008); in another study the counts remain constant (Talon *et al*, 2007); in Greek sausages, the counts were reduced gradually, regardless of their initial concentration (Drosinos *et al*, 2005). Factors that may influence the growth of *Enterobacteriaceae* during sausage fermentation include a high initial a_w; a high initial pH value; a low concentration of fermentable carbohydrates; low numbers of lactobacilli in the fresh sausage mixture and high ripening temperatures. During drying, *Enterobacteriaceae* including *Salmonella* (Gonzalez-Fernandez *et al*, 2003, Fernando-Lopez *et al*, 2008, Porto-Fett *et al.*, 2008; Dourou *et al.*, 2009; Hwang *et al*, 2009; Lindqvist and Lindblad., 2009) and *Yersinia* spp., are slowly inactivated (Hajmeer *et al.*, 2011).

Enterohaemorrhagic strains of *Escherichia coli* (EHEC)

Strains of the bacterium *E. coli* capable of producing certain cytotoxins are reported as verotoxigenic *E. coli* (VTEC). Enterohaemorrhagic *E. coli*, commonly referred to as EHEC, are a subset of the VTEC, harbouring additional pathogenic factors. More than 150 different serotypes of VTEC have been associated with human illness; however, the majority of reported outbreaks and sporadic cases of VTEC infections have been attributed to serotype O157:H7. There is a wide spectrum of symptoms associated with VTEC infections, ranging from mild to bloody diarrhoea, often accompanied by severe abdominal cramps but usually without fever. VTEC infection can also result in haemolytic uremic syndrome (HUS). HUS is characterized by acute renal failure, anaemia, and lowered platelet counts. HUS develops in up to 10% of patients infected with VTEC O157 and is

the leading cause of acute renal failure in young children (Skandamis, 2007). Severe outbreaks of haemorrhagic colitis attributed to the consumption of fermented sausages occurred in western states of the USA and Australia (Tilden, 1996; CDC, 2000). The infective dose of the causative agent is generally low, and multiplication during fermentation is not required to cause disease. Moreover, inactivation during sausage ripening may be slow (Calicioglu *et al.*, 1997, 2001, 2002; Dourou *et al.*, 2009); reductions of 1 to 5 logs were observed, mainly dependent on pH, water activity, ageing time and temperature (Cosansu and Ayhan., 2000; Pond *et al.*, 2001 Porto-Fett *et al.*, 2008; Hwang *et al.*, 2009; Hajmeer *et al.*, 2011). The reduction of verotoxigenic *E. coli* (VTEC) (O157:H7 and O103:H25) by process and recipe optimisation in dry-fermented sausages, was studied by Heir *et al.* (2010).

Staphylococcus aureus

Staphylococcus aureus is of significant practical importance in meat. Not only can this bacterium cause a variety of infections in meat animals, as well as in humans, but it is also the causative agent of a major form of food poisoning. The most common symptoms are nausea, vomiting, retching, abdominal cramping and prostration. In more severe cases, headache, muscle cramping and transient changes in blood pressure and pulse may occur (Skandamis, 2007). Bad practices during manufacturing, especially poor conditions of fermentation (deficient hygiene, too high temperature, no pH control) and poor conditions of storage (deficient hygiene, improper temperature control), have led to food-poisoning

outbreaks from products, especially those that are consumed raw (Skandamis, 2007). *Staphylococcus aureus* is frequently found in raw meat and fermented sausages, but generally at low levels. Only if allowed to grow to cell densities above 10^5CFU/g, does it form levels of enterotoxins sufficient to cause illness. The bacterium is little affected by salt, but is a poor competitor under anaerobic conditions, at low pH values and low temperatures. Several authors have studied the ability of *St. aureus* to grow in fermented sausages (Nychas and Arkoudelos.,1990; Gonzalez-Fandos *et al.*, 1999).

Spore forming bacteria

Raw sausage mixtures may contain considerable numbers of *Bacillus* and *Clostridium* spores of which spices are a major source. Reviews of the production of cured meats with respect to the risk of botulism and the risk for outgrowth of all foodborne spore-formers (Cerveny, 1980) pointed out many factors that prevent growth and toxin production by these pathogens. No one factor in cured meats completely inhibits the growth of all pathogens. Rather, a combination of a mild heat treatment that sensitizes cells to curing agents, different salts and curing substances and a low pH, act to keep the foods safe (Doyle *et al.*, 2001). Although the germination of bacterial spores is prevented by salt or saturated brine preservation, these spores will remain present and develop into vegetative cells when conditions are more favourable (Wijnker *et al.*, 2011). Growth of *C. botulinum* and neurotoxin production are controlled by nitrite, low pH, and low a_w values in these products (Peck and Stringer., 2005).

Listeria monocytogenes

Listeria monocytogenes, the causal agent of listeriosis, is a Gram-positive, rod-shaped and psychrotrophic pathogen which is widely distributed in the environment and consequently present in various animal products and in vegetables (Erkmen., 2008). A further special feature is its osmotolerance being able to survive in the presence of high salt concentrations (up to 20% w/v) and low a_w (0.91) (Lado, 2007).

In humans, infections most often affect the pregnant uterus, the central nervous system, or the bloodstream. Symptoms vary, ranging from mild flu-like symptoms and diarrhoea to life threatening infections characterized by septicaemia and meningoencephalitis. In pregnant women, the infection spreads to the foetus, which will either be born severely ill or die in the uterus, resulting in abortion. Although human infections are rare, it is worth noting that it is considered to be a high mortality type of infection. For non-invasive listeriosis to occur in the general population, ingestion of foods with levels of contamination greater than 10^5 CFU/g appear to be required (NZFSA, 2009). The illness is usually evident within two days of consuming contaminated food. By contrast, in vulnerable consumers, the invasive form can occur after ingestion of as few as 100-1000 cells and the symptoms of illness may not be seen for as long as 90 days, but usually around 30 days. This makes linking the illness to a particular food difficult (NZFSA, 2009). Generally, enteric illnesses are under-reported, because patients experiencing only mild symptoms or discomfort may choose not to visit a health

care provider, and those who do visit a physician do not always provide a faecal sample. In fact, for every case of enteric illness reported, many hundreds of cases may go unreported, increasing the difficulty of accurate surveillance (Luber *et al.*, 2011).

Control of other biological hazards

Moulds and mycotoxins

Originally, the so-called *house flora* just contaminated the product, but when the significance of mycotoxins was realized, development proceeded to avoid the use of toxigenic moulds. Particularly in the south European countries, dry sausages are applied with atoxigenic yeast and moulds to produce products with specific flavour notes. This is done by dipping or spraying. Mould cultures tend to suppress natural moulds and, consequently, reduce the risk of mycotoxins production. The ripening techniques considered positive and normally applied in industrial practice usually allow for the growth of characteristic whitish moulds, which can also inhibit the multiplication of other moulds, especially those that prove to be potentially toxinogenic and/or with a mycelium having undesirable color (Baldini *et al*., 2000)

Biogenic amines

Biogenic amines (BA) are organic bases with aliphatic, aromatic or heterocyclic structures that can be found in several foods, in which they are mainly produced by microbial decarboxylation of amino acids, with the exception of physiological

polyamines (Silla Santos *et al.*, 1998). At high concentrations, BAs may cause hypotension, hypertension, nausea, rash, dizziness, increased cardiac output and increased respiration (Bover-Cid *et al*, 2003; Bandolin *et al.*, 2010). High amounts of amines can be found in fermented foods derived from raw materials with high protein content, such as dry sausages. Tyramine and putrescine are the most common BA found in dry sausages and their presence is often due to the activity of LAB (Bover-Cid *et al.*, 2003; Suzzi and Gardini, 2003; Bandolin *et al.*, 2010). High amounts of cadaverine and, to a lesser extent, of histamine, have been detected in some samples and related to the low quality of raw materials. During the processing of dry fermented sausages, the ripening step allows favourable conditions for the formation of BA. There is growth of microorganisms for a period of time, during which a certain degree of proteolysis takes place producing free amino acids as precursors of BA and also, usually the pH drops slightly, favouring the synthesis and activity of amino acid decarboxylases of bacterial origin (Bover-Cid *et al.*, 2001). When considering a protective culture, it is fundamental to guarantee the quality of final products in relation to their BA content. For this reason, the inability to form BA, to survive during ripening and storage and to possess amine oxidase activity should be relevant criteria to be taken into consideration in the selection of protective cultures for the fermentation of dry sausages (Suzzi and Gardini, 2003).

1.2 *Listeria monocytogenes*

1.2.1 Regulatory control in ready-to-eat products

There is a statutory obligation for Member States to report cases of human listeriosis to the European Union (EFSA) as part of the Zoonoses Directive (EU, 2003), but is still not in practice in Portugal. The European Food Safety Authority and the European Centre for Disease Prevention and Control (EFSA-ECDC) summary report of zoonoses in the European Union, in 2009, refers to a rate of human listeriosis of 0.36 cases per 100,000 population (EFSA, 2009). The number of confirmed cases increased by 19% in 2009 (n=1,645) compared with 2008 (n=1,381) (Lahuerta *et al*, 2011). A case fatality rate of 19% was reported in people aged 65 years and over). The highest notification rate was also reported in this age group (1.1 cases per 100,000 population), representing 59% of all reported cases. Only 4.2% of the reported cases were detected among children aged under five years. In 2009, the highest proportions of non-compliant food products at retail level were found in RTE fish products, cheese (especially soft and semi-soft cheeses) and RTE products of meat origin, although the level was lower than in the previous two years. The high proportion of deaths among elderly people as a result of *Listeria* infection is of particular concern**.** In developed countries where listeriosis has been documented and regulations for its control have been in place over many years, the incidence rate of human infections seems very similar (0.3 to 0.7 per 100,000 population) with slightly higher rates in northern European countries (Todd and Notermans, 2011). The incidence of

reported listeriosis ranges from 0.5 per 100,000 population in New Zealand (NZFSA, 2009), 0.26 cases per 100,000 of the US population (CDC, 2011.) and Canada with 0.2 cases per 100,000 of the population (Warriner *et al*, 2009). In 2007, Finland reported 0.8 cases per 100,000, France reported 0.46 cases per 100,000, Germany 0.4 cases per 100,000, Iceland 1.3 cases per 100,000, Luxembourg 0.6 cases per 100,000, Norway 1.0 cases per 100,000 and Sweden 0.6 cases per 100,000 (EFSA, 2009). France, the United Kingdom and several other European countries have also reported increases in the incidence of listeriosis over the last several years. In these countries, the increase has been predominantly driven by an increased incidence in patients over 60 years of age (Ross *et al*, 2009c). The large increase in European cases in listeriosis has been attributed to the ageing population who are susceptible to acquiring the pathogen (Goulet *et al*, 2008). Dalton *et al*. (2004) noted that outbreaks, in Australia between 1995 and 2000, in aged-care and hospital settings were associated with 35% of all deaths but with only 5% of outbreaks and less than 3% of cases. The minimum infectious dose for this risk group is not known, though it is assumed to be high for most healthy adults (Todd and Notermans, 2011). In Portugal, listeriosis is not a reportable disease, and has, thus, been underestimated. Recent evidence indicates that human listeriosis occurs in Portugal at levels similar to those encountered in other developed countries (Almeida *et al.*, 2006, 2010). According to these authors, 35 cases of listeriosis were identified in the period between 1994 and 2003 inclusive, the mortality rate being greater than 17%. According to this study, the incidence of this disease in Portugal was at least 0.14

cases per 100,000 inhabitants for the year 2003, increasing to 0.23 cases per 100,000 inhabitants for the year 2007. Based on current case data from around the world, the likelihood of a food contaminated with low numbers of *L. monocytogenes* resulting in illness is considered to be remote (FAO/WHO, 2004). Foods containing low levels of *L. monocytogenes* (e.g., < 100 CFU/g at the point of consumption) pose very little risk (Chen *et al*., 2003; FAO/WHO, 2004). In fact, in instances where foods linked to listeriosis outbreaks were still available for testing, the levels of *L. monocytogenes* detected both from unopened foods and leftover foods obtained from the patients, have usually been high (i.e., $>10^3$ CFU/g), and thus these outbreaks were due to non-compliant samples. Consequently, a lower priority should be placed on products in which the organism cannot grow or, has a limited potential for growth whereby the levels do not exceed 100 CFU/g throughout the stated shelf-life. Recently, the European Commission re-examined *L. monocytogenes* regulatory policies. EC Regulation 1441/2007 (EC, 2007) requires that in ready-to-eat foods that will support the growth of *L. monocytogenes* during their normal storage conditions, this pathogen must be undetectable in 25 g of the product, while under the control of the manufacturer, and may be detectable in concentrations inferior to 100 CFU/g while on the market during their shelf-life (EC, 2007). Only ready-to-eat foods for infants and special medical purposes are excluded from this criterion: in these products, *L. monocytogenes* should be absent in 25 gram samples; in products that do not support growth, concentrations inferior to 100 CFU/g while on the market during their shelf-life are allowed. The *Codex Alimentarius* Commission,

in its 2007 "Guidelines on the Application of General Principles of Food Hygiene to the Control of *L. monocytogenes* in Ready-To-Eat Foods" (CAC, 2007) also considers the application of different microbiological criteria for RTE foods depending on the possibility of growth of *L. monocytogenes* in the product. Also, a risk-based approach to sampling for *L. monocytogenes* is considered mandatory (CAC, 2007; Luber *et al*, 2011). Some countries, like the United States, practice 'zero tolerance' (no organisms found in 25 g of a food product) (McLauchlin *et al.*, 2004), while Canada adopted criteria nearer those of the European Union. In Australia and New Zealand, *Listeria* regulations have been developed that are applicable to the product at any point in the shelf-life of the product, in a risk-based approach. These countries establish limits according to the nature of the food and its ability to support growth, considering high, medium and low risk food groups, reflecting the Codex Commission on Food Hygiene. Zero-tolerance (not detected in 25 g sample) applies to foods with a previous link to listeriosis or the ability to support the growth of *L. monocytogenes*. High risk products have been identified as unpasteurized milk and dairy products (including butter, soft or semi-soft cheeses), deli meats, cooked pasta, paté and cooked seafood (NZFSA, 2009).

1.2.2. *Listeria monocytogenes* in dry fermented meat products

This organism can contaminate meat and meat products during slaughter, processing and production, can persist and grow at low and high pH values, at low a_w and at refrigeration temperatures, and may cause serious food safety problems

for consumers (Tyopponen *et al*., 2003; Colak *et al*., 2007; Thévenot *et al*., 2005a). Fermented meat products are often contaminated with *Listeria* and are produced without any lethal processing step, but the final composition can prevent growth of *L. monocytogenes* during storage (FAO/WHO, 2004). *Listeria* spp. and *L. monocytogenes* were detected in fermented sausage "Sukuk" (Aksu and Kaya, 2004; Colak *et al*, 2007);, Italian style sausage, "Chouriço", cured ham, salami, (Guerra *et al.*, 2001); "alheira" (Ferreira *et al.*, 2006, 2007b, Esteves *et al.*, 2008), "salpicão" and "chouriça" (Ferreira *et al.*, 2007a). *L. monocytogenes* was detected in a variety of other meat products such as fermented sausages (Cantoni *et al*, 1989; Farber *et al*., 2000b, Levine *et al.*, 2001, Jemmi *et al*., 2002). The pathogen has also been found in fermented Italian fermented sausages (De Cesare *et al.*, 2007). Another study on Portuguese sausage "linguiça" showed the presence *Listeria* spp. and *L. monocytogenes* (Silva *et al*., 2004).

1.2.3. Inhibition of growth of *Listeria monocytogenes* in dry fermented meat products

As previously referred, the traditional dry fermented smoked sausage process itself is able to reduce contamination, although to a variable extent. In regard to *L. monocytogenes*, several studies have evaluated the effectiveness of sausage manufacturing processes to control this pathogen, with considerable variability, probably due to the different process parameters used in each study (Nightingale *et al*., 2006). Inactivation of pathogens during maturation/ripening is, in addition to the control of initial pathogen levels and growth, a crucial step in the safe production of fermented sausages not undergoing heat treatment (Encinas *et*

al.,1999; Drosinos *et al.*, 2005; Hew *et al.*, 2005; 2006; Porto-Fett *et al.*, 2008; Lindqvist *et al.*, 2009; Montet *et al.*, 2009), though these may not be completely efficient for this purpose (Thévenot *et al.*, 2005b; Nightingale *et al.*, 2006; Montet *et al.*, 2009). Concern about the resistance of emerging pathogens to conventional food preservation techniques and consumer resistance to chemical forms of control, are increasing private and public research interest in expanding the opportunity for layers of protection, a "multi-hurdle" approach, rather than relying on a single method (Tyopponnen *et al*. 2003, Arnau *et al*, 2007). There has been great interest in methods of biological preservation (bio-preservation). One strategy being the subject of studies is the use of a natural bacteriophage, LISTEX™ P100, with antagonistic effect against *L. monocytogenes*, and classified by the FDA as GRAS (generally regarded as safe), for all food products. Other possibilities include the use of protective LAB and/or their antibacterial products such as bacteriocins, to enhance food safety. Bacteriocins are defined as biologically active proteins with antibacterial properties (Tagg *et al*., 1976; James *et al*., 1991). The most commercially important bacteriocin is nisin, produced by *Lactococcus lactis*. Nisin has been shown to be effective in a number of food systems, inhibiting the growth of a wide range of Gram-positive bacteria, including important foodborne pathogens such as *L. monocytogenes*. Bioprotective cultures may act as starter cultures in the food fermentation process, such as dry sausage manufacturing and may also include live bacteriocin-producing LAB in the process, with the purpose of protecting foods without affecting organoleptic characteristics. Recently, the use of cultures to produce

bacteriocins *in situ* as a means of biopreservation has been receiving increasing attention. Lactic Acid Bacteria that produce bacteriocins with antilisterial activity have been used in fermented sausages as starter cultures (Schillinger *et al*., 1991; Foegeding *et al*., 1992; Nieto-Lozano *et al.*, 1992; Campanini *et al*., 1993; Hugas,, 1998; Mataragas *et al.*, 2003; Matilla *et al.*, 2003; Ananou *et al.*, 2005; Teixeira de Carvalho *et al.;* 2006, Alves *et al.*, 2006; Héquet *et al.*, 2007; Hampikyan and Ugur, 2007; Albano *et al*, 2009; Ruiz-Moyano *et al*, 2009, 2011; Bello *et al*, 2010**;** Hadji-Sfaxi *et al*, 2011; Castro *et al*, 2011). The LAB species most commonly used to control the growth of *L. monocytogenes* in fermented sausages are *Pediococcus acidilactici*, *Lb. sakei*, and *Lb. curvatus*. *Pediococcus acidilactici* and the *Lactobacillus* spp. are the main species used as starter cultures for the manufacture of American and European fermented sausages, respectively, because they are able to out-compete other organisms during meat fermentation (Hugas, 1998). It is an undisputed requirement that, besides their adequate antimicrobial ability, protective cultures should not adversely affect the food. They should not significantly influence the sensory properties of the product they are used in (Casquete *et al.*, 2011; Ruiz-Moyano *et al.*, 2011), and also should not possess virulence factors nor resistance to antibiotics. The bioprotective cultures should not be capable of producing significant levels of biogenic amines (Bover-Cid *et al.*, 2001; Pereira *et al.*, 2001, Latorre-Moratalla *et al.*, 2010; Komprda *et al.*, 2010**;** Curiel *et al.*, 2011; Baka *et al.*, 2011; Casquete *et al.*, 2011), D(-)-lactic acid and other toxic metabolites and undesirable compounds in food stuffs. L(+)-lactic acid is more inhibitory than its D(-) isomer (Benthin and Villadsen., 1995).

The D(-) isomer is not hydrolyzed by human lactate dehydrogenase and may cause health problems, so only strains producing predominately L(+) lactic acid should be selected (Buckenhüskes, 1993; Holzapfel, 2002; Ammor and Mayo., 2007). It is essential to verify if cultures used in food products possess traits which might contribute to their virulence. Several genes coding for virulence factors in *Ec. faecalis* have been characterized (Table 1.4.) and their effects have been studied in animal models (Chow *et al.*, 1994; Huycke *et al*., 1992; Schlievert *et al.*, 1998, Martín-Platero *et al.*, 2009, Hadji-Sfaxi *et al.*, 2011). Other enzymes such as gelatinase, DNase, lipase and the ability to form biofilms are also traits that contribute to *Ec. faecalis* virulence in some animal models (Qin *et al*., 2000; Seno *et al*., 2005). There is little information about these virulence factors in other LAB, though they are important factors to be evaluated. Research by Ruiz-Moyano *et al*. (2009), on lactobacilli for potential probiotic use in Iberian dry-fermented sausages, showed their low aminogenic potential and D-lactic acid production, antibiotic resistance pattern and haemolytic activity. Since the high load of endogenous bacteria often present in raw meat will contact with starter bacteria, the probability of genetic transfer of antibiotic resistance might represent a problem in meat fermentations (Cocconcelli *et al*., 2003; Gevers *et al*., 2003a,b; Ruiz-Moyano *et al*, 2009). Recently, some studies have focused on the application of additional hurdles such as bacteriocins and/or high hydrostatic pressure (HHP), in the control of of *Salmonella*, *L. monocytogenes* and *St. aureus.* (Jofré *et al.*, 2009; Ananou *et al.*, 2010).

Table 1.4. *Enterococcus* virulence factors identified

Gene(s)	Role of product in virulence	References
agg	Aggregation protein involved in adherence to eukaryotic cells; cell aggregation and conjugation	Galli *et al*. (1990)
***gel*E**	Toxin; extracellular metallo-endopeptidase, hydrolyzes gelatin, collagen, haemoglobin, and other bioactive compounds	Su *et al.* (1991)
***cyl*Lv** ***cyl*Ls**	Cytolysin (haemolysin-bacteriocin) precursor; expression of *cyl*Lv-Ls, -M, -B, and -A is required for production of active cytolysin which lyses a broad range of eukaryotic and Gram positive cells	Gilmore *et al.* (1994)
***Cyl*M**	Post-translational modification of cytolysin	Gilmore *et al.* (1994)
***Cyl*B**	Transport of cytolysin	Gilmore *et al.* (1994)
***cyl*A**	Activation of cytolysin	Gilmore *et al.* (1994)
***es*p**	Cell wall-associated protein involved in immune evasion; may be associated with *cyl* genes on a pathogenicity island.	Shankar *et al*. (1999)
efaAfs ***efaAfm***	Cell wall adhesins expressed in serum by *Ec. faecalis* and *Ec. faecium*, respectively	Singh *et al*. (1998)

Adapted from Eaton *et al* (2001).

High hydrostatic pressure processing is a non-thermal method that has shown potential in producing microbiologically safer products while maintaining the natural characteristics of the food items (Omer *et al.*, 2010; Rendueles *et al.*, 2011). As indicated above, several studies have been published concerning

bacteriocin-producing LAB being successfully used to control *L. monocytogenes* in meat products.

1.3 Quantitative Microbial Risk Assessment

1.3.1. Background and components of Quantitative Microbial Risk Assessment

Prior to the mid 1970's, food safety was neither a significant political, scientific or societal concern (Cooter and Fulton, 2001). One of the first recorded large scale food safety incidents occurred in southern France in 944A.D., when 40,000 people died of ergotism, a disease caused by substances (alkaloids) termed ergotamines produced by *Claviceps purpurea* that contaminates rye and wheat products (Schiff, 2006). In the last few decades, there have been several food safety issues, at national, European, and international level, associated with spiralling public anxiety and escalating media attention, that have resulted in a decline in public confidence in food safety regulation and management inside and outside Europe (e.g. Houghton *et al*., 2008). Situations such as bovine spongiform encephalopathy (Reilly, 1999), dioxins (Verbeke, 2001), genetically modified foods (Frewer *et al*., 2004), and acrylamide (Claus *et al.*, 2008), are some of the most media-led scares that exacerbated and distorted public awareness regarding the risk for their health. Consequently, these events result in short-term negative impacts upon consumer consumption/purchase behaviour as well as negative impacts upon the producer, manufacturer or retailer (Knowles and Moody, 2007).

Minimising food-borne pathogens has been a key objective for most European countries. Hazard Analysis Critial Control Points principles were instated as a food safety requirement in E.U., through EU Directive 93/43. A key response to European food scares has been the creation of institutions responsible for the implementation and verification of food standards (Oslo Renard,2005). In almost every EU country, government motivations for the creation of such agencies are attributed to declining consumer trust (Poppe and Kjaernes, 2005). The European Commission also identified food safety as a key policy priority in response to the BSE crisis, and in 2004 the European Food Safety Authority (EFSA) was created. Whether EU food policy evolved to primarily protect human health or was rather aimed to restore consumer confidence was a question tackled between the European Parliament, the European Commission and the European Council (Knowles and Moody, 2007).

In 1991, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) communicated that transparent, science-based and internationally recognised standard approaches to risk assessment were needed and that these should be consistently applied across the board of committees establishing such assessments in different discipline domains. The agreement in the World Trade Organization and in particular the Sanitary and Phytosanitary agreement (WTO-SPS) (WTO, 1995) created a worldwide need for science-based standards for food safety and established health risk as the only basis for restrictions of international trade in food (Ross and McMeekin, 2003). The ‘‘High Level International Food Safety Forum” (Beijing, China; 26-27

November 2007) re-affirmed the need for transparent food safety regulations, based on risk analysis (Havelaar *et al.*, 2008). The WTO relies on the *Codex Alimentarius* to define the safety of food and to specify how Risk Analysis, and particularly Risk Assessment, should be performed.

The process of decision-making applied to risk has been termed ‘‘risk analysis.’’ Risk Analysis is a framework proposed for governmental bodies to define an appropriate level of public health protection and establish guidelines to ensure the supply of safe foods (Gorris *et al.*, 2002). Risk Analysis, as shown in Figure 2, is composed of the three elements Risk Assessment, Risk Management and Risk Communication (FAO/WHO 1997; EFSA, 2006b).

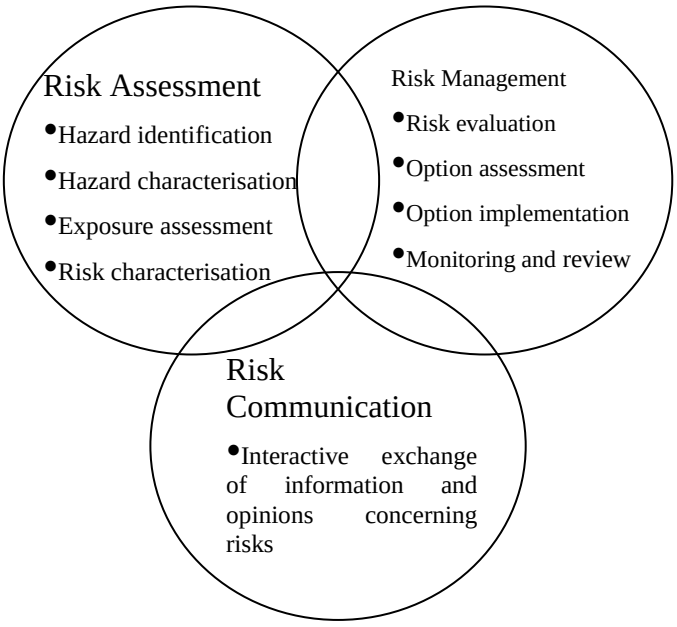


Fig. 2: *Codex Alimentarius* Commission schematic for Risk Analysis. Diagram illustrates the interaction between each of the three main components comprising Risk Analysis. (FAO/WHO 1997)

Every person assesses risk on a daily basis. Every time one crosses a wet floor, one estimates the likelihood of falling and the extent of damage it could do. If the

estimated risk is small enough, the person starts to walk, because he or she has decided that the risk, although not zero, is acceptable and that the benefit (i.e. not going around) outweighs the risk. Risk assessment of foods is basically the same process. The risk assessor estimates the risk of harm consequent to an action (consumption of a certain food), based on existing information and using different techniques. The estimate of risk is then evaluated in order to reach a decision. The decision may be to accept the risk and inform the consumer, or to implement actions to reduce the risk to a tolerable level (zero risk does not exist). Such decisions on risk level have mainly been taken by governments, though certain aspects of risk assessment can also be used by food safety managers in industry (Marvin *et al.*, 2009).

1.3.2. Microbial Risk Assessment

Two forms of risk assessment, chemical risk assessment and ecological risk assessment provided the foundations for Microbiological Risk Assessment, (MRA) (Reij and Schothorst, 2000; Parkin, 2008). Many issues in MRA differ importantly from chemical risk assessment, demanding different conceptual and practical modelling approaches (Haas *et al.*, 1999; Parkin, 2002; Eisenberg,2006; FAO/WHO, 2003; Schaub, 2004; WHO, 2005). Some of the problems that researchers (Parkin, 2008) have identified include:

- Microorganisms can grow, evolve into different life stages, and die off.
- Virulence varies during a pathogen's life cycle and between different pathogen strains.

- Pathogens behave differently under different temperature and time conditions (e.g., in food processing and preparation), as well as in different media and matrices (e.g., different types of foods).
- Microbial pathogens are not evenly distributed in the environment, and may be found in clumps, which present very uneven probabilities of exposure.
- Secondary or person-to-person transmission occurs in many infectious disease processes, while chemical exposure assessment occurs in the individual.
- Attack rates and infection rates differ and asymptomatic carriers exist.
- Multiple, independent exposures occur as part of the infectious disease process

The most widely adopted framework for MRA derives from the chemical risk assessment framework described by a four-step model—involving hazard identification, dose-response and exposure assessment, and risk characterization (NRC, 1983; CAC, 1997; FAO/WHO, 1997) in a four step process elaborated below:

1. Hazard identification: In MRA, a hazard is a biological agent, with the potential to produce an adverse effect in an individual (Lammerding and Fazilb, 2000).

2. Exposure assessment: Exposure is a function of the quantity of a food consumed and the level of contamination, meaning concentration and prevalence in that food at the time of consumption (FDA/CFSAN, 2003). Also, it is described as an estimate of how likely it is that an individual or a population will be exposed to a microbial hazard and what numbers of the microorganism are likely to be ingested (Lammerding and Fazilb, 2000).

If a quantitative exposure assessment is conducted, simulation modelling and data collection may predominate in this step (Rocourt *et al*., 2001). Most of the time, sufficient data is not available, as the frequency of contamination of foods and total number of pathogens ingested by consumers is not known (FAO/WHO, 2004). It is more likely that data on prevalence and concentration is available from previous steps in the food chain, hence, an estimate can be calculated using mathematical models (Walls, 2006). Predictive microbiology can be used to estimate changes in microbial numbers throughout the food chain, in a "farm-to-fork" approach, and allows exposure to a particular pathogen to be assessed. All assumptions, sources of data and uncertainties must be clearly stated and identified (CAC, 1999). Exposure assessment modelling techniques are fast developing to account for pathogen dynamics in food at different temperatures and under different conditions.

3. Hazard characterisation: This may be a qualitative and/or quantitative evaluation of the nature of adverse health effects associated with the hazard (Rocourt *et al*., 2001). This step estimates the magnitude of illness for an individual or a population, based on exposure to the pathogen(s). The microbial dose-response is a model that characterises the relationship between the dose of the organism and an adverse effect given by that dose. This effect or end point may be infection, disease, illness or even mortality (Latimer et *al*., 2002). Traditionally, dose–responses were described in terms of a single value such as an LD50 or ID50 value. This led to the concept of minimum infectious dose, i.e. the

minimum number of bacteria needed to cause disease. There has been substantial effort to define the minimum infectious dose for various food-borne pathogens. These efforts have typically not been successful (Buchanan *et al.*, 2000). However, a number of mathematical models have been used to describe the entire sigmoidal dose-response curve (McLauchlin *et al.*, 2004). Several dose response models have been described in the literature, including the log-normal, log-logistic, Weibull-gamma (with consumption of soft cheese made from raw milk, by Bemrah *et al*, (1998), exponential and beta-Poisson (Holcomb *et al*., 1999), Weibull and Gompertz (Buchanan *et al.*, 2000). One important issue is the extrapolation of the model from the population on which it was developed, often animals, to the population under consideration. This extrapolation applies not only to the mathematical form of the dose-response relationship, but also to the values of the parameters in that relationship (McLauchlin *et al.*, 2004). An alternative hypothesis is that if one considers a large enough cross section of the human population, the ingestion of a single pathogenic bacterial cell has a finite possibility of causing an infection, and that this probability increases as the levels of the pathogen increase (Haas *et al.*, 1983). The use of the exponential curve in the development of dose-response assessment is recommended by the draft ‘‘WHO/FAO Guidelines on Hazard Characterization for Pathogens in Food and Water for the selection of dose–response models for infectious microorganisms’’, (FAO/WHO, 2003). This has been adopted in official Risk Assessments for *L. monocytogenes*, developed by the FDA (FDA/CFSAN, 2003) and later, in the WHO/FAO report in 2004.

4. Risk characterisation: This is the qualitative and/or quantitative determination or estimation of the probability of occurrence and severity of the investigated adverse effect in a given population, including uncertainties, using the above listed steps (Rocourt *et al.,* 2001). The information from the above steps is synthesised to produce a risk estimate. This output is severely affected by the quantity and accuracy of information input into previous steps (Walls, 2006). The degree of confidence in the risk estimate depends on variability and uncertainty identified, as well as the assumptions made. These must be well documented and undergo re-evaluation, as new relevant information becomes available (CAC, 1999).

1.3.3. Quantitative microbial risk assessment

In this section, basic principles, types of model, simulations, sampling techniques, and statistical analyses will be addressed.

1.3.3.1.Mathematical models

The objective of modelling is to make a simplified representation of reality (Zwietering and Besten, 2011). The use of mathematics represents in a simplified way an existing biological system by mathematical modelling. Hence, models permit easy, swift and cost efficient predictions of how a biological system will behave and allow the quantification of dynamic biological functions. However, the effectiveness of models to describe the biological system depends on the quantity and quality of the data available, the accuracy and detail of the model

structure and the current knowledge. These models can be used to perform various tasks from quantitatively describing a phenomenon, testing significant kinetic differences, quantitatively investigate mechanisms and correlations, designing experiments and sampling plans, or predicting phenomena within a food chain to enable optimal control (Zwietering and Besten, 2011,). The mathematical model uses a set of assumptions that will result in mathematical equations (Zwietering and Besten, 2011). Primary models describe the change of the bacterial number over time under determined environmental conditions and generate information about the microorganism such as generation time and lag phase duration. Secondary models describe the evolution of one or more parameters of a primary model in relation to one or more changes in environmental conditions. Tertiary models take modelling to its final form. They are applications to one or more primary or secondary models, incorporated into a user-friendly computer software package.

1.3.3.2. Deterministic and probabilistic models

Mathematical models can be classified as deterministic or probabilistic. In the deterministic or point estimate approach, for a given set of initial conditions, a series of point estimates, e.g. the average, or the worst case (Lammerding and Fazilb, 2000), exactly the same output will be predicted. Variables are defined by fixed point values. This produces a model in which each step is calculated algebraically to produce a single point value estimate (Guevara *et al.*, 2011). In probabilistic modelling, all the possible values of a parameter are used, probability distributions of the input variables are used rather than a single value

point estimate (Carrasco and Angelidis, 2007; Koutsoumanis and Angelidis *et al.*, 2007). Stochastic is derived from the Greek word, *stokhastikos*, meaning "capable of guessing". Probabilistic models permit the inclusion of the natural variation inherent to biological systems, into models. Variables are described by probability distributions, describing the range of values may occur and the respective frequency or the probability. In deterministic models, both the inputs and outputs are expressed as single numbers or point values. These may represent a "best guess", the "average" or "expected case" or perhaps the "worst case". When one wants to determine the impact of one or more of the input values on the output, one simply substitutes a new value into the model (OIE, 2004). However, the microbial quality and safety may be characterized by a (high) level of variation and, as such, deterministic models provide limited information for risk analysis studies (Poschet *et al.*, 2003). If average values are considered, the extremes are disregarded, and these high values, representing a pathogen concentration like *L. monocytogenes*, may represent the high risk portion for the population (Buchanan, 2003). Deterministic models are therefore limited in their ability to report a range of values over which the event occurs. Nevertheless, they are useful for simple models with few inputs, to give the risk manager an estimation of risk, in a preliminary analysis.

Probabilistic models are applicable with more complex models, or in situations where one has more data to work with. When information about the range of values and the likelihood of each value is available, it is possible to assign a probability distribution to each variable; these can now be described as random

variables as they can take on a different value as a result of a random process. Such a model is called a probabilistic or stochastic model and it is possible to calculate the combined impact of the variation in each of the model's input distributions to determine a probability distribution of the possible model outcomes. The simplest way to do this is to perform a simulation. This involves randomly sampling values from each distribution and combining the values generated, according to the mathematical model, to produce a result for that particular scenario. This process is repeated several times and the results from each scenario, which are also known as iterations, trials or realisations, are combined to produce a probability distribution of possible model outcomes. Probabilistic models take into account the entire distribution of possible values, incorporating both uncertainty and variability. Uncertainty represents the lack of perfect knowledge about the modelled system, be it from errors with respect to the parameters, or simplification of realistic processes, which can be reduced by further measurements or data collection (Nauta, 2000; Lindqvist *et al.*, 2002; Mokhtari and Frey, 2005). Model uncertainty may occur as a result of over-simplification of modelled processes and the use of inappropriate or unknown variables, while parameter uncertainty can be a consequence of measurement, sampling and/or systematic errors. Variability represents the true heterogeneity of the population that is a consequence of the physical system and it is irreducible by additional measurements (Nauta, 2000, 2002). Both variability and uncertainty can be described using probability distributions. The separation of variability and uncertainty model parameters is critical in producing an accurate risk estimate

(Nauta., 2000; Pouillot *et al.*, 2003, 2007). For safe food production, evaluation of risk may be less important, as it 'just' means to keep the risk low. For public health objectives, risk has to be evaluated quantitatively, both for reasons of comparison with other health risks and for the evaluation of proposed risk mitigation strategies (Nauta, 2000). A risk assessment therefore has to incorporate probabilities throughout the analysis (Nauta, 2002).

1.3.3. 3. Simulation modelling

Simulation is a technique that calculates a model output multiple times with different input parameters. The aim of this technique is to get a complete range of all possible outcomes. Simulation modelling is simplified through the use of computer programs designed to perform multiple iterations of a mathematical model from a particular set of input values. After the model is constructed, the software calculates all of the possible combinations of factors by calculating the answer many times. Each outcome is called an iteration. A single simulation consists of one to any number of iterations. The result of a single simulation incorporates all the outputs of every iteration within that simulation. All of those values generate a distribution of possible outcomes. Because some or all of the independent variables in the model are characterized by a range of possible values, there is a range of outcomes, some of which will occur more often than others (WHO, 2004*).*

1.3.3. 4. Monte Carlo simulation

Monte Carlo analysis consists of the random selection of a single 'point-estimate' value from each of the probability distributions assigned for each input parameter. The randomly selected single values are used to calculate a mathematical solution defined by the risk assessment model, and the result is recorded. Values that are more likely to occur, according to the defined probability distribution, are selected more frequently (Lammerding and Fazilb, 2000). This simulation technique is widely used in Quantitative Microbiological Risk Assessment (QMRA) modelling and has several advantages. Monte Carlo methods are useful for modelling phenomena with significant uncertainty and variability in inputs, which we assume is the case in most farm-to-fork models. These methods are especially useful in studying systems with a large number of coupled degrees of freedom, i.e. correlation and other inter-dependencies are modelled in a very natural manner. This means that a very complex domain can be modelled using Monte Carlo simulations, and is easy to implement using commercial software. The distributions of the model's dependent variables do not have to be approximated in any way, because they are obtained by simulations (Smid *et al.*, 2010). However, Monte Carlo simulation has some weaknesses: there is no interactive link between data and parameters, it is unidirectional, hence there is no interaction between the different parts of the model, validation is often *ad hoc* (by means of re-sampling) and it does not allow "backwards reasoning" (Smid *et al.*, 2010). Its accuracy is directly proportional to the number of iterations performed in each simulation. This can be time-consuming (Poschet *et al.*, 2003). Another disadvantage is that

this technique performs poorly when modelling rare events (Vose *et al.*, 2001). Other methods of calculating outcome distributions for QMRA exist, such as methods of moments and exact algebraic solutions. These are, however, not as commonly used as Monte Carlo simulations.

1.3.3. 4.1 Simulation sampling

There are several different methods of sampling when using Monte Carlo simulations, but only two of the more commonly used methods will be discussed.

1.3.3. 4. 1.1 Monte Carlo sampling

The Monte Carlo method is based on simple random sampling from the entire distribution, which represents the sampling frame for each iteration. It is sampling with replacement, as it is possible for the same values to be selected more than once (OIE, 2004).

1.3.3. 4.1. 2. Latin hypercube sampling

Latin hypercube sampling, on the other hand, involves stratified sampling without replacement. The range of the distribution is divided up into a number of intervals, equal to the number of iterations to be performed and a simple random sample is then chosen from within each interval. Each interval is only selected once during a simulation. As a result, Latin hypercube sampling ensures that values from the entire range of the distribution will be sampled proportionally to the probability density of the distribution. Fewer samples are usually required to reproduce the probability distribution so it is more efficient than Monte Carlo sampling for the same number of iterations. It is generally the preferred method of

numerical simulation since fewer iterations are required for a particular level of accuracy (OIE, 2004).

1.3.3.4.1.3 Sensitivity analyses

It can be tempting to include every phenomenon that could be of relevance, but this could lead to over-parameterized models (Zwietering., 2009). Sensitivity analysis can help solve this issue by eliminating the less influential factors. Sensitivity analysis of models are mathematical techniques designed to identify the parameters most influential to the model output and therefore the investigated processes (Ellouze *et al.*,, 2010). If a model is highly sensitive to a parameter in which there is some degree of concern about its accuracy, this indicates that more information is needed on the parameter to increase the model's robustness and practical value. Generally, knowledge of key inputs describing variability in the model can help to identify efficient control measures, while knowledge of key inputs describing uncertainty highlights areas that require further data collection (Ellouze *et al.*, 2010). Sensitivity analyses can therefore be of great assistance to risk managers, responsible for the development of targeted control strategies (Frey and Patil, 2002).

1.3.4. Second order modelling

Second order modelling is the separation of variability and uncertainty, which allows a more correct risk estimate to be produced (Nauta, 2000; Dawber *et al.*, 2009; Busschaert *et al.*, 2011). As previously stated, uncertainty refers to a lack of knowledge of data, that can be reduced by acquiring more relevant data, whereas

variability represents the true heterogeneity of the population that is irreducible and unaffected by more data. The incorporation of both uncertainty and variability in a single risk assessment distribution can result in a loss of information regarding the individual contributions of each of these two components. Furthermore, failure to separate the two can lead to difficulty in the interpretation of the output, or worse, to meaningless outputs. Vose (2001) identified two methods to separate uncertainty and variability. One calculates variability and simulates uncertainty, while the other simulates variability, selecting a random sample from the uncertainty distributions for each simulation. Nauta *et al*. (2003) separated the two by implementing Monte Carlo simulations first sampling from uncertainty distributions, and then sampling from variability distributions. This method separately propagates uncertainty and variability in risk assessments by nesting multiple realisations of model parameters and iterations of input variables. The output is a collection of distribution functions describing the uncertainty and variability in the results (Dawber *et al.*, 2009). Second order Monte Carlo simulations are also known as two-phase or two-dimensional Monte Carlo simulations.

1.3.5 The concept of Food Safety Objectives in the frame work of QMRA.

The appropriate level of protection (ALOP) and correlated concepts were introduced in the World Trade Organization Agreement on the Application of Sanitary and Phytosanitary Measures (the SPS Agreement), which promotes the use of risk assessment, based on objective and accurate scientific data, when

setting food safety standards (ICMSF, 2002). Food Safety Objectives (FSOs) determine the maximum frequency and/or concentration of a hazard in a food at the time of consumption that provides or contributes to the ALOP. FSOs constitute the link between the ALOP and food industries (Havelaar *et al.*, 2004; Andersen and Nørrung, 2010, Whiting *et al.*, 2011). Risk managers are responsible for setting the ALOP (Walls, 2006). To achieve the FSOs, Performance Objectives (POs) (maximum frequency of occurrence (%) and/or concentration (CFU/g) of a pathogen) at other stages, Performance Criteria (PC) change (i.e. reduction or maximally allowed increase) in frequency of occurrence and/or concentration of a pathogen that should be achieved during processing or implementation of control measures should be established in the process prior to consumption. Governmental risk managers are responsible for establishing ALOP and FSOs, whereas industrial risk managers should design production processes to meet the FSOs (Walls, 2006). Compliance of ALOP, FSO, POs or PC should be based on data and findings originating from scientific resources and/or studies such as Quantitative Microbiological Risk Assessment. A QMRA study produces a wealth of information useful for risk assessors and risk managers. It can be used as a tool to collect information regarding the microbiological hazard under study. Then, the food safety manager can use this information to design the production process, apply control measures and risk management in general (Perez-Rodriguez *et al*., 2006; Walls, 2006; Whiting *et al.*, 2006, Whiting 2011). However, it should be borne in mind that so far no FSOs have been set by food safety managers. A QMRA study may give valuable information regarding the

presence and development of a microbiological hazard in a food product. The people involved in food safety may use this information to draw conclusions, publish directives relative to risk management or establish POs and/or PC. The QMRA model can be used as a baseline to evaluate the effectiveness of different risk management options or control measures (i.e. "what-if" scenarios) (Fels-Klerx *et al*,, 2008; Schothorst *et al.,* 2009; Mataragas *et al.,* 2010; Sosa Mejia *et al.*, 2010).

1.3.6 *Listeria monocytogenes* quantitative microbial risk assessments

This section summarizes the frameworks that different organizations around the world used to conduct microbial risk assessments.

In the United States, to date, the Food and Drug Administration (FDA), with the Center for Safety and Applied Nutrition (CFSAN) have produced a Risk Assessment of *L. monocytogenes* in ready-to-eat-foods (FDA/CFSAN, 2003). This risk assessment organized food products into categories. Models were used to estimate levels of individual and public health risks for each category in three subpopulations (perinatal, elderly and intermediate age). In Canada, health risk assessment for *L. monocytogenes* in foods concentrated on pâté and semi-soft cheese, was performed following *Codex Alimentarius* guidelines. The dose response curve for *L. monocytogenes* infections was derived using the Weibull-Gamma model. Normal and high-risk populations were considered (Farber *et al.*, 1996). Australia has elaborated a QMRA for *L. monocytogenes* in Australian Processed Meats. The model used distinguished three types of processed meat

products: luncheon meats, pâtés/liverwursts and cooked sausages. This QMRA included the effect of LAB in the pathogen growth, through the "Jameson" effect. The dose-response model used is that presented in FAO/WHO (2004) (Ross *et al.*, 2009 a, b). New Zealand elaborated a Risk Profile intended to help inform the NZFSA *Listeria monocytogenes* Risk Management Strategy, and to provide a scientific underpinning for associated risk management measures (Lake *et al.*, 2009). To date, EFSA has not been in a position to carry out Quantitative Microbiological Risk Assessment (QMRA) and the scientific opinions of the Panel on Biological Hazards (with the exception of those on BSE/TSE) are mainly based on qualitative and in some cases semi-quantitative risk assessment (EFSA, 2007; Hugas *et al.*, 2007; Havelaar *et al.*, 2008). The FAO/WHO *Listeria monocytogenes* in ready-to-eat foods risk assessment reflects the state of knowledge as at 2002 (FAO/WHO, 2004). Four foods were selected for the risk assessment, each different in terms of contamination, storage and consumption patterns: milk, ice cream, cold-smoked fish and fermented meat products. The probability of becoming ill from ingesting *L. monocytogenes* is higher for susceptible populations compared to the general population. Based upon the US data, people aged over 60 years were 2.6 times more susceptible relative to the general healthy population. Perinatal neonates were 14 times more susceptible. The same analysis has been carried out on other susceptible sub-populations. The quantitative data on *L. monocytogenes* contamination of food was based primarily on European foods, the consumption data were based on Canada or the USA (FAO/WHO, 2004). Giovaninni *et al.* (2004) have performed the risk assessment

of listeriosis due to consumption of Parma ham, according to international guidelines set by FAO /WHO (2004).

CHAPTER 2. MATERIALS AND METHODS

2.1 Microbial characterization of "Salpicão de Vinhais" and "Chouriça de Vinhais"

2.1.1 Origin and sampling:

2.1.1.1. Final product:

Fourteen samples of "Salpicão" and thirteen of "Chouriça" were purchased from four different local producers, at their plant. Seven samples of each product were purchased in a local market, in Vinhais. The results obtained for the product, in the last week of processing (see 2.1.1.2) were also considered, in a total of 16, for each type of "Traditional dry fermented, smoked sausage" (TDFSS). These 57 samples were analyzed for each item described in 2.1.2. Ten samples of ''Salpicão'' and ten samples of ''Chouriça', from different producers were randomly collected at retail stores. These samples were only analysed for detection of *L. monocytogenes* and detection of *Y. enterocolitica*. For each parameter to be evaluated, unless otherwise stated, two independent analyses were performed using randomly selected pieces.

2.1.1.2. During processing:

Two samples from two producers, A and B, were collected at different stages during production, directly from industrial factories located in the North of Portugal.

Samples from two different batches were analysed, during the study. Samples were taken immediately and after 48 h of seasoning at 4 ºC, after stuffing into casings before smoking; and weekly, during the smoking process, until the end of production. A total of 48 samples of "Salpicão" and 40 samples of "Chouriça", from both producers A and B, were collected for analysis (5 or 4 weeks of processing for "Salpicão" or "Chouriça", respectively x 2 duplicates per batch x 2 batches x 2 producers).

2.1.1.3 Raw materials:

Samples from the raw materials (pork meat, garlic, laurel, paprika, salt and salted pork casings) were collected from two different processors, A and B.

All samples were transported to the laboratory under refrigeration (approximately 0 ºC, in melting ice) and were analysed within 24 h.

2.1.2 Microbiological Analyses

2.1.2.1 Indicator and pathogenic bacteria

Twenty-five gram samples were added to 225 mL of sterile buffered peptone water (Merck, Darmstadt, Germany), and homogenized in a stomacher for 2 min.

Appropriate decimal dilutions were prepared in sterile Ringer's solution (LabM, Bury, UK) for microbial enumeration: Aerobic mesophilic counts on Plate Count Agar (LabM) incubated aerobically at 30 ºC for 72 h, according to ISO 4833 (ISO, 2003); *Enterococcaceae* on bile esculin azide agar (Biokar Diagnostics, Beauvais, France), incubated at 30 ºC for 72 h; yeasts and moulds on rose-bengal agar supplemented with 0.1 g/L of chloramphenicol (Oxoid, Hampshire, UK), incubated at 25 ºC for 5 days; *Escherichia coli* on TBX (BioRad, Hercules, CA, USA), incubated at 44 ºC for 24 h; coagulase-positive staphylococci on Baird-Parker RPF-agar (bioMérieux, Marcy l'Etoile, France), incubated at 37 ºC for 48 h, according to NF V08 057-2 (AFNOR, 2004); sulphite reducing *Clostridium* spores according to the Portuguese Standard NP 2262 (Anonymous, 1986).

Enumeration of *Listeria* spp. was performed by direct plating on PALCAM agar medium (Merck) and by the Most Probable Number (MPN) technique using culture media referred to in ISO 11290-1 (Anonymous, 1996b), namely Demi-Fraser broth (Merck), Fraser broth (Merck) and PALCAM medium. Positive results were confirmed according to the International Standard ISO 11290-2 (Anonymous, 1998). Detection of *L. monocytogenes* was performed using the VIDAS method (Anonymous, 1996a), an enzyme-linked fluorescent immunoassay performed in the automated VIDAS instrument, using antibody specific for *L. monocytogenes* and also by direct enumeration according to the ISO 11290-2 (Anonymous, 1998). *Salmonella* spp. were also detected by the VIDAS method (Anonymous, 1994). Positive results were confirmed using as enrichment broths Rappaport–Vassiliadis (bioMérieux) and Muller–Kauffmann

Tetrathionate (bioMérieux), according to the standard techniques described in ISO 6579 (Anonymous, 2002). Detection of presumptive pathogenic *Y. enterocolitica* was performed as described in ISO 10273 (Anonymous, 2003), following three successive stages: (1) enrichment in peptone, sorbitol and bile salts (PSB) broth and in irgasan™, ticarcillin and potassium chlorate (ITC) broth (Sigma-Aldrich, Germany); (2) surface plating on solid selective culture media, namely agar with cefsulodin, irgasan™ and novobiocin (CIN) (Sigma-Aldrich, Munich, Germany) and *Salmonella* / *Shigella* agar, with sodium desoxycholate and calcium chloride (SSDC) (Sigma-Aldrich). Presumptive tests such as urea-indole, Kligler and oxidase were carried out on suspected characteristic colonies, being small, smooth with a red centre and translucent rim, very finely granular when examined with obliquely transmitted light.

2.1.2.2 Lactic acid bacteria

Twenty-five grams of sample was added to 225 mL of sterile buffered peptone water (Merck), and homogenized in a stomacher for 2 min. Appropriate decimal dilutions were prepared in Ringer's solution (LabM) for microbial enumeration. A volume of 1.0 mL of each dilution was plated onto MRS agar (LabM) and M17 agar (LABM), respectively, and incubated at 30 ºC for 72 h, under microaerophilic conditions, using an air-tight container and depleting oxygen with a burning candle flame. Representative colonies were picked from MRS and M17 plates, showing 15-150 colonies. Isolates were obtained by repeated streaking onto solid growth media. Preliminary characterization of isolates comprised

colony and cell morphology, Gram stain, oxidase and catalase tests (Norris *et al*., 1981). Gram-positive, catalase and oxidase negative isolates were selected for further studies.

Working cultures were stored at -20 ºC in MRS broth or M17 broth, depending on the isolation media, with 30% (v/v) glycerol added, and sub-cultured twice before being used in assays. Stock cultures were stored at -80 °C in MRS broth or M17 broth, depending on the isolation media, with 30% (v/v) glycerol.

Strains of LAB previously isolated from "Salpicão" and "Chouriça", and described by Ferreira *et al.* (2007a) were also considered for this study.

2.2 Physico-chemical characterization of "Salpicão de Vinhais" and "Chouriça de Vinhais"

Chemical analyses were performed on final product samples and samples taken during the smoking process as described in section 2.1.1.

2.2.1 pH, sodium chloride and moisture content

pH was determined directly with a Crison MicropH 2002 pH-meter (Crison, Barcelona, Spain) equipped with an InLab 427 puncture electrode (Mettler Toledo, Columbus, OH, USA).

Chloride and moisture contents were determined following the ISO Standards 1841-2 (Anonymous, 1996b) and 1442 (Anonymous, 1997), respectively.

2.2.2 Water activity

The water activity was measured with a Hygroplam AW1 (Rotronic Instrument Corporation, USA).

2.2.3. Temperature

Temperature inside one "Salpicão", one "Chouriça" and room temperature were recorded in two different producers, A and B, using a temperature datalogger HANNAH Instruments Model HI98804 (USA).

Temperature during transportation and storage was also recorded inside one "Salpicão", one "Chouriça" from the plant to retail store, from producer A.

2.2.4 D-lactic acid, L-lactic acid concentration

D - Lactic acid / L - Lactic acid in the samples of raw meat, samples of semi-processed product and final product were determined enzymatically by using D - lactate and L - lactate dehydrogenase kit (Catalog No. 1112821035, Boehringer Mannheim Roche, Germany). Extraction of D/L lactate from the meat samples was performed by accurately weighing approximately 5 g, adding approximately 20 mL of perchloric acid and homogenizing for 10 minutes. The mix was quantitatively transferred into a beaker with approximately 40 mL of water. The pH was adjusted to 10-11 with potassium hydroxide 2M, while stirring (magnetic stirrer). Afterwards, the contents were quantitatively transferred into a 100 mL volumetric flask with water washings, was and then filled up to the mark with water. Care was taken as to the fatty layer being above the mark and the aqueous

layer at the mark, as this is the fraction of interest. The flask was placed in the refrigerator for 20 minutes to promote fat separation and precipitation of potassium perchlorate. The next step was filtration, discarding the first few millilitres.

In the presence of D - lactate dehydrogenase (D-LDH); D - lactic acid (D-lactate) is oxidized to pyruvate by nicotinamide-adenine dinucleotide (NAD^+). The oxidation of L - lactic acid requires the presence of the enzyme L - lactate dehydrogenase (L-LDH) (1 and 2)

$$\text{D-lactate} + NAD^+ \xleftrightarrow{D-LDH} \text{pyruvate} + NADH + H^+ \qquad \text{(Eq. 1)}$$

$$\text{L-lactate} + NAD^+ \xleftrightarrow{L-LDH} \text{pyruvate} + NADH + H^+ \qquad \text{(Eq. 2)}$$

The equilibrium of these reactions lies on the side of lactate. By trapping pyruvate in a subsequent reaction catalyzed by the enzyme glutamate-pyruvate transaminase (GTP) in the presence of L-glutamate, the equilibrium can be displaced in favour of pyruvate and NADH (Eq. 3).

$$\text{Pyruvate} + \text{L-glutamate} \xleftrightarrow{GPT} \text{L-alanine} + \alpha\text{-oxoglutarate} \qquad \text{(Eq. 3)}$$

The amount of NADH formed in the above reactions is stoichiometric to the amount of D - lactic acid and L - lactic acid, respectively. The increase in NADH is determined by means of its light absorbance at 340 nm.

2.3 Risk assessment methodology:

The methodology of microbiological risk assessment adapted the conceptual framework used in the risk assessments of *L. monocytogenes* in ready-to-eat foods, (FDA/CSFAN, 2003; WHO, 2004), as described in Fig. 2.1.

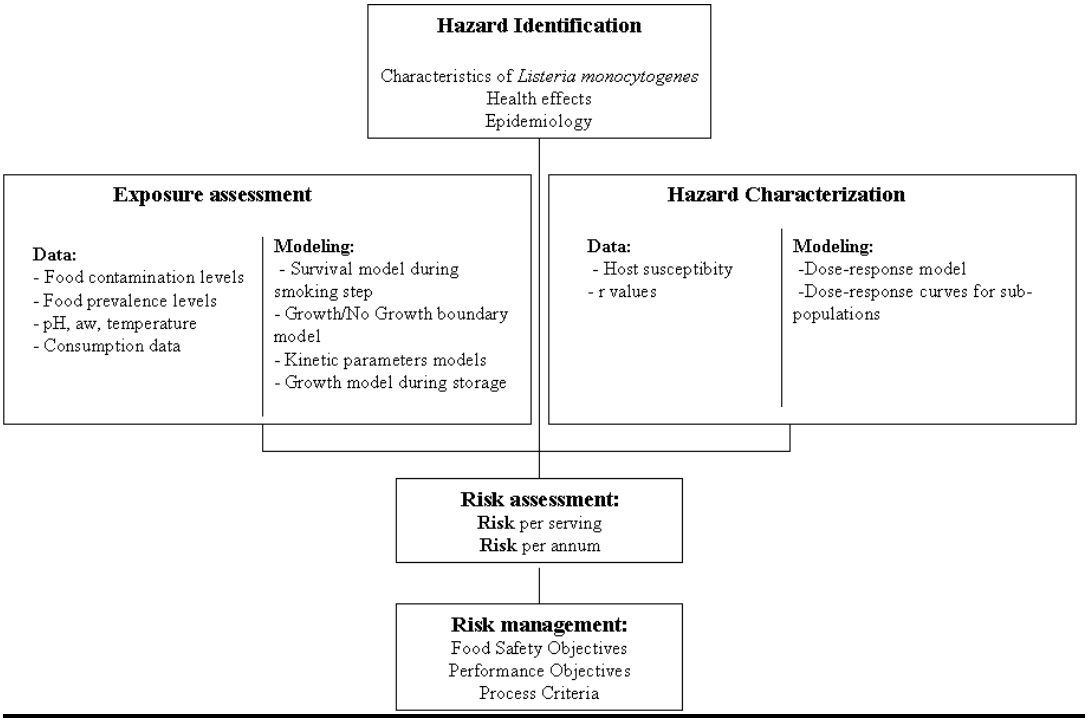


Fig. 2.1: Overview of *Listeria monocytogenes* risk assessment for traditional dry fermented meat products. Adapted from (FDA/CFSAN, 2003)

2.3.1 Exposure Assessment

The model used in this study is a contamination assessment model for *L. monocytogenes* in dry, semi-dry fermented meat sausages (Koutsoumanis and Angelidis, 2007). The model is presented in Fig. 2.2 and is composed of a secondary model, which calculates the effects of the environmental conditions (pH, temperature, and water activity), on the probability of growth or no growth. If there are conditions for growth, the same environmental conditions are used to calculate the lag time and maximum growth rate, μ_{max}. The parameters are then used in a primary growth model, which computes the final contamination level,

$N_{predicted}$, given the secondary model output (lag time, μ) and the initial contamination level N_i.

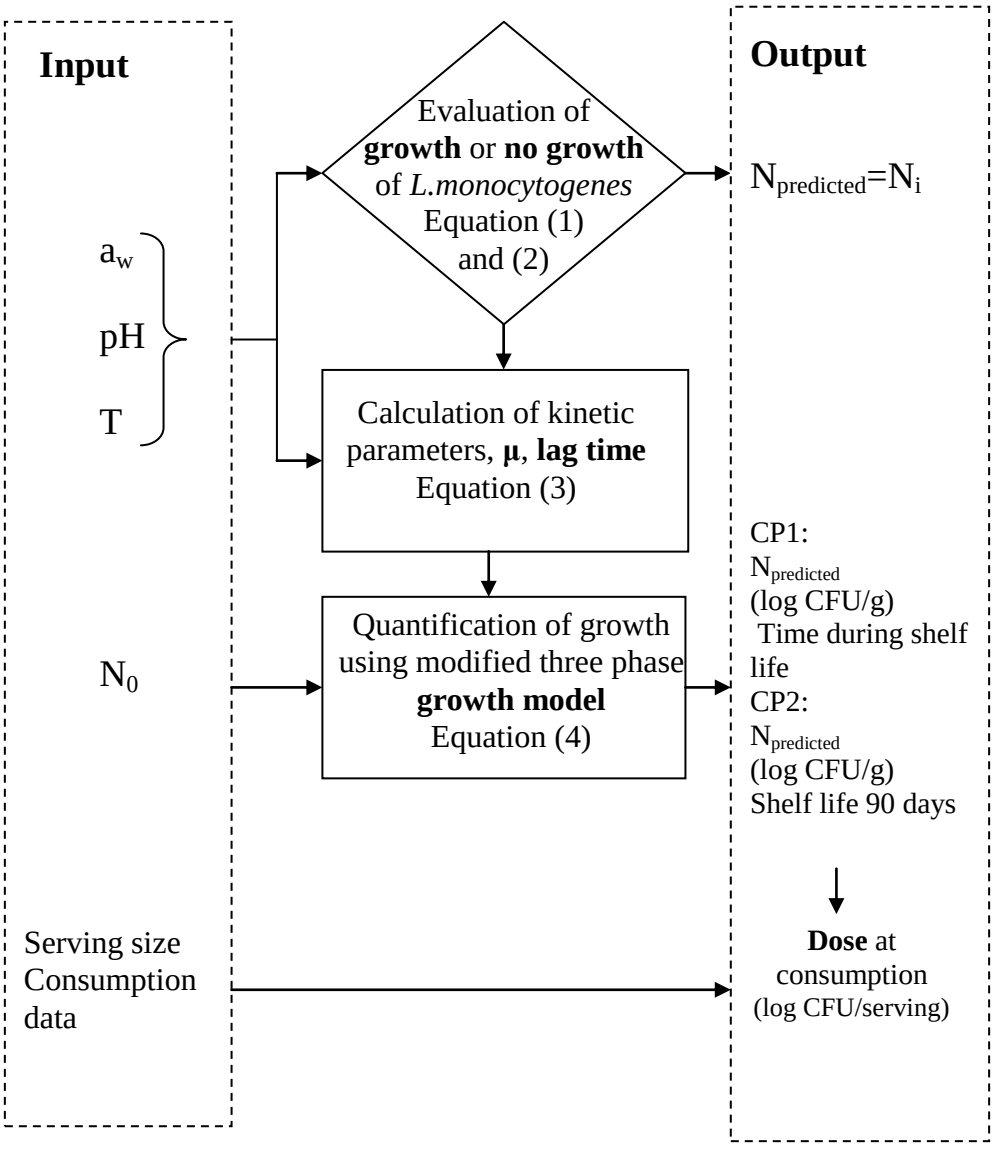


Fig. 2.2: Components of the Exposure Assessment Model.

2.3.1.1 Estimation of the distribution frequency of contamination with *L. monocytogenes*

The prevalence and concentration of *L. monocytogenes* in the final product were determined as previously described in section 2.1. Two types of data, describing the of contamination in the samples, were obtained:

- Presence/absence (qualitative) data (i.e., the number of positive samples relative to the total sample collection).
- Enumeration (quantitative) data (i.e., the number of colony forming units (CFU) of *L. monocytogenes* that were measured from a sample).

The available data on *L. monocytogenes* levels had some limitations that affected the distribution for levels of the pathogen in food. First, there are relatively few data points above the limit of detection (0.04 CFU/g). This is because there are few classical microbiological analytical results in which a count of *L. monocytogenes* was possible (below detection limit) and negative analytical results in classical microbiology techniques were further investigated, by immunoassay, for the occurrence of *L. monocytogenes* rendering either positive or negative samples.

It is assumed that one CFU represents one organism (FDA/CFSAN, 2003).

According to the literature (Kilsby and Pugh,1981; Gill *et al*., 1996; Giovaninni *et al*., 2007), the detected contaminated samples arise from a continuous log normal distribution of contamination (Fig. 2.3) , and the minimum detectable level from the presence/absence tests is typically 1 organism in 25 g or 0.04 organisms per gram. A low percentage of samples are contaminated at or above this level with

the remaining samples having non-detectable levels (i.e., < 0.04 organisms per gram).

Data from presence/absence analysis (qualitative data) were converted to numerical data and included in the model by assigning the lowest possible contamination level that can be detected by the laboratory method. For a method that uses a 25 g sample, the lowest detectable level is 0.04 CFU per gram of food. In this way, the qualitative data could be used along with the quantitative data in the construction of the cumulative distribution curves of *L. monocytogenes* levels in food (FDA/CSFAN, 2003; Giovannini *et al*, 2007).

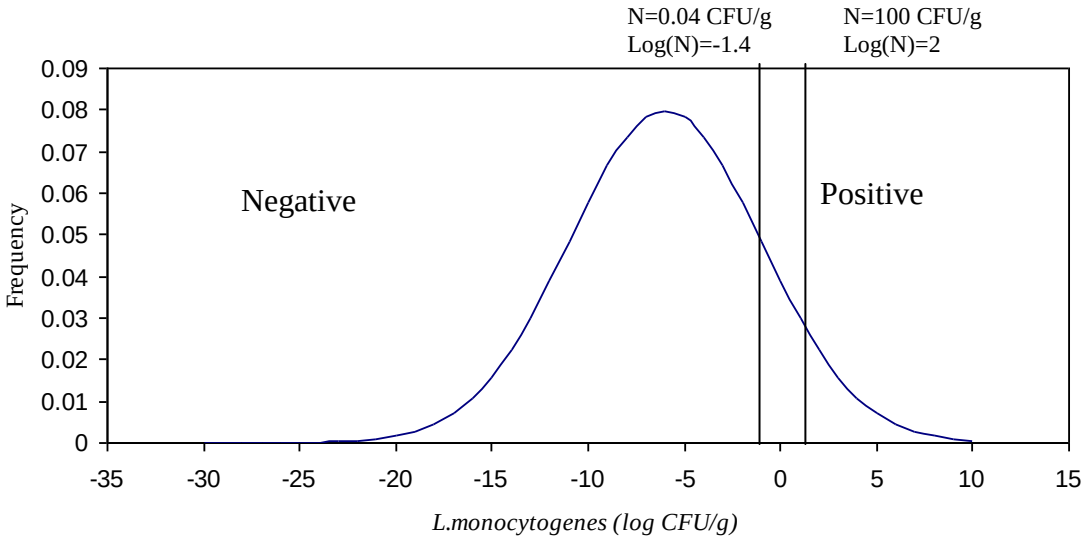


Figure 2.3 Example of the log-normal contamination curve showing frequencies of detectable and non detectable samples (adapted, FDA 2003).

All *L. monocytogenes* food isolates were considered to be homogeneous regarding their potential to cause human illness, ability to grow or other characteristics (FDA/CSFAN, 2003).

2.3.1.1.1 Estimation of the mean and standard deviation of lognormal distribution of N_i

A log-normal curve with the appropriate standard deviation was fitted to the presence/absence data by "sliding" the mean until the percentage of positive samples corresponded to the presence/absence data. To estimate the mean and the standard deviation of the log normal distribution, wide ranges of values for the mean and for the standard deviation have been considered and the best fitting pair of values has been chosen as that minimizing the D statistics in the one-sample Kolmogorov– Smirnov test for goodness-of-fit (Giovannini *et al*, 2007):

$$Max(D_{\mu_j,\sigma_j}) = Max\left(\left|\int_{-\infty}^{x_i} Normal(x_i,\mu_j,\sigma_j) - \sum \frac{observed_i}{N}\right|\right) \qquad \text{(Eq.5)}$$

where D is the Kolmogorov–Smirnov statistics; x_i is each measured contamination level; μ_j the hypothesized mean; σ_j the hypothesized standard deviation; $Observed_i$ the number of samples showing that specified contamination level, and N the total number of samples (Giovannini *et al*, 2007).

2.3.1.2 Estimation of the distribution frequency of final product characteristics (pH, a_w, temperature profile)

The frequency histogram of pH, a_w and temperature were built and the distribution function that best described this was selected, using @Risk 4.5 (Palisade Corporation Software, New York, NewYork, USA, 2005)

2.3.1.3 Mathematical models

2.3.1.3.1 Modelling the growth/no growth boundary of *Listeria monocytogenes*

Giving the pH and a_w characteristics of the final products tested, it was considered important to develop a model of Growth/No Growth within the parameters of pH, a_w and temperature as similar as possible to the actual conditions of this product (Mataragas *et al.*, 2006, Porto-Fett *et al.*, 2008).

2.3.1.3.1.1 Growth medium preparation.

Two series of experiments were performed. First, several solutions of tryptone soy broth with no sodium chloride were prepared and pH was adjusted to different values (5.0, 5.4, 5.8, 6.2, 6.6) with 1M lactic acid solution (Vaz Pereira, Porto, Portugal). Aliquots of 40 mL from the resulting solutions were divided into 100 mL flasks and sodium chloride was added to obtain different water activities ranging from 0.86 to 0.94 (simulating the actual a_w of the products under study). The pH and the a_w were as described in 2.2.1 and 2.2.2, respectively. The solutions were autoclaved at 121 °C for 15 min. The a_w and pH values were measured again to check for possible deviations from values of pre-autoclaved solutions, after cooling to ambient temperature (25 °C). The afore-mentioned pH and a_w values as well as those further presented in the results, are those measured after autoclaving. Aliquots (225 μL) of each experimental combination were added to each single well of a 96-well microplate and all wells were inoculated with 25 μL of the *L. monocytogenes* culture. Enumeration on Triptyc Soy Agar (TSA) by inoculation with 1.0 mL by spread plating with broth from these wells and incubated for 24 h at 37 °C showed that the average inoculum concentration

in microtiter plate wells was 2.7 to 3.9×10^4 CFU/mL. All microplates were sealed with parafilm to prevent evaporation and incubated at different temperatures according to the experimental design. (Koutsoumanis and Sofos, 2005)

2.3.1.3.2 Experimental design

A full factorial design with three factors was used to evaluate the effect of acid adaptation in broth and on the growth/no growth interface of *L. monocytogenes* strain NCTC 10527 (from the National Collection of Type Cultures, London, UK). The factors examined were incubation temperature (5, 10, 15, 25 °C), pH (5.0, 5.4, 5.8, 6.2, 6.6) adjusted with lactic acid, and a_w (0.96, 0.94, 0.92, 0.88, 0.86) achieved by the addition of NaCl. The exact a_w was determined by constructing a calibration curve of a_w against added salt concentrations in Triptyc Soy Broth and Yeast extract (TSBYE). E. pH was adjusted with lactic acid before autoclaving, at 121ºC for 15 min, and measured and adjusted again after autoclaving by aseptically removing small volumes. Each combination was studied in triplicate samples, by using three microtiter plates for each temperature. Three wells in each plate were filled with non-inoculated sterile Triptyc Soy Broth (TSB) as a non-contaminated control. The cultures were kept at 4 to 10 °C to ensure that growth was minimized until the inoculated broth preparations were dispensed aseptically into wells in sterile microtiter plates.

2.3.1.3.3 Evaluation of growth/no growth.

Growth was recorded when a visible increase in the turbidity of the broth in a well, was observed. When the results were doubtful, possible growth was recorded and the well was re-examined subsequently to confirm growth based on further increases in turbidity. Turbidity was assessed by visual inspection and was recorded daily for approximately the first 7 days, and thereafter turbidity was assessed weekly for up to 60 days, when the experiment was ended.

When growth was not clear, after 60 days of incubation, two TSA plates were inoculated by 1.0 mL spread plating with broth from these wells and incubated for 24 h at 37 °C. Growth was considered as present when the population of the potential positive well increased, by at least 0.5 log CFU/mL above the initial inoculation level (Skandamis et *al.*, 2007).

2.3.1.3.4 Evaluation of the ability of traditional dry, fermented, smoked sausages to support the growth of *Listeria monocytogenes*

i) Growth/No growth interface model and binomial transformation

The experimental data was translated into binary values of 0 and 1, corresponding to no-growth or growth, respectively, and fitted to a logistic regression model according to the method of Ratkowsky and Ross, (Ratkowsky and Ross, 1995; Koutsoumanis and Angelidis, 2007), using the StatGraphics Plus 2.1 computer-based program (Statistical Graphics Corporation, Warrenton, Virgínia, United States). The variables considered were temperature, a_w and pH. Lactic acid was

not considered directly since it was used to adjust pH. So, the total effect of lactic acid was indirectly described through the pH term.

For modelling purposes, a_w was converted to b_w, calculated as the square root of $1- a_w$ (Gibson *et al.*, 1987). The following equation describes the model used:

$$\text{Logit}(Pg) = a0 + a_1T + a_2pH + a_3a_w + a_4TpH + a_5Ta_w + a_6pHa_w + a_7T_2 + a_8pH_2 + a_9 a_w^2 \quad \text{Eq. (1)}$$

where, Logit (Pg) is an abbreviation of ln[Pg/ (1−Pg)], Pg is the probability of growth (in the range of 0–1), a_i are the unknown coefficients of the equation, and T is temperature. The Hosmer-Lemeshow statistic was used to measure the goodness-of-fit of the developed model (Mataragas *et al*, 2006).

The probability of growth of the pathogen, in the serving, at a given consumption point, was calculated by treating the data on the probability of growth derived from the Monte Carlo simulation as a binomial random variable with the parameter *Pg*:

$$\text{If binomial } (1, Pg) = \begin{cases} 0 & \text{the sausage is unable to support growth} \\ 1 & \text{the sausage is able to support growth} \end{cases} \quad \text{Eq. (2)}$$

A binomial variable only takes two possible values, in this case, given the temperature, a_w and pH conditions, the sausage is able to support growth (1) or not (0).

Dry/semi-dry fermented sausages are considered to be unable to support growth of *L. monocytogenes* (FDA/CSFAN, 2003). However, the traditional

manufacturing process is highly variable and empirical. Equations (1) and (2) translate the possibility that traditional dry/semi dry fermented smoked sausages might be able to support growth of *L. monocytogenes*, depending on the final pH and a_w of the products. Through equation (2), if the probability of growth is higher than 50%, the binomial output is 1, otherwise it is 0 (Koutsoumanis and Angelidis, 2007). If the binomial output is 1, then growth may occur and the exponential growth rate and lag phase may be calculated, as described below.

ii) Calculation of exponential growth rate (μ) and lag phase

Equations (3a) and (3b), that represent the full quadratic response surface models for generation time and lag phase duration for the aerobic growth of *L. monocytogenes* (*Buchanan and Phillips,* and 2000) are used in the equation (4):

$$\ln(GT) = 227.7984 - 0.2465T - 380.8103\ a_w + 0.00829TpH + 0.0308\ NaNO_2 - 0.0287T\ a_w + 0.00829TpH - 0.0000025TNaNO_2 + 3.0406\ a_w\ pH - 0.0111\ a_w\ NaNO_2 - 0.00268pHNaNO_2 + 0.00274T_2 + 174.7631\ a_w^{\ 2} + 0.388pH^2 + 0.0000003NaNO_2^2 \quad \text{Eq.(3a)}$$

Where GT is the generation time ($GT=\log(2)/\mu$) in hours.

$$Ln(lag) = 252.833 + 0.1418T - 358.21\ a_w - 18.4395pH + 0.0151NaNO_2 - 0.3653Ta_w + 0.00452TpH + 0.0000169TNaNO_2 + 11.8359a_wpH + 0.00437a_wNaNO_2 - 0.00269pHNaNO_2 + 0.00201T^2 + 132.4864a_w^2 + 0.4881pH^2 + 0.0000005NaNO_2^2 \quad \text{Eq. (3b)}$$

The terms related to the concentration of $NaNO_2$ were not considered as the products follow the traditional formulation, with no addition of preservatives.

iii) Calculation of growth (Buchanan et *al.*, 1997)

$$N_t \begin{cases} N_i & \text{for } t \le t_{lag} \\ N_i + \alpha\mu(t-t_{lag}) & \text{for } t_{lag} < t < t_{max} \\ N_{max} & \text{for } t \ge t_{max} \end{cases} \qquad \text{Eq. (4)}$$

The output of the Eq.(1) and Eq.(2), 0 or 1, is then multiplied by the output of Eq. (4), which is the calculation for growth (that uses the following Eq. (3a) and Eq. (3b), meaning that if the pH and a_w do not allow growth, then the initial contamination remains, N_i, but if the combination of pH, T and a_w are such that it is considered to allow growth of the pathogen, then a N_f is calculated. Predicted growth was calculated at two distinct consumption periods (CPs). The two distinct CPs were a hypothetical period during storage of products as determined by a normal distribution (CP1), and the end of a shelf life of 90 days (CP2) (Koutsoumanis and Angelidis, 2007).

2.3.1.4 Calculation of final concentration of *Listeria. monocytogenes* by probabilistic modelling approach

The distribution of the concentration of *L. monocytogenes* at the end of the shelf life was calculated based on the above modelling procedure using a Monte Carlo simulation technique with @Risk software 4.5 using Latin Hypercube sampling.

Ten thousand (10,000) repetitions (iterations) in each simulation of the model were conducted.

The structure of the model and variables used in the simulation model are summarized in Fig. 2.2. Model inputs describe temperature, pH, a_w, and initial concentration of *L. monocytogenes*. The distributions of pH, a_w and T were derived by fitting the experimental data with the distribution function that gave the best fit, using program @Risk software 4.5. The output of this simulation is the distribution of predicted growth of *L.monocytogenes* at each control point, CP1 and CP2 (Drosinos *et al*, 2005, Koutsoumanis and Angelidis, 2007).

2.3.2 Hazard Characterization

Hazard characterization represents the qualitative and quantitative evaluation of the nature of the adverse effects.

2.3.2.1 Dose–response assessment

The conceptual model adopted for the Hazard Characterization is summarized in Fig.2.4. The dose-response assessment was based on an exponential curve as recommended by the draft ‘‘FAO/WHO Guidelines on Hazard Characterization for Pathogens in Food and Water for the selection of dose–response models for infectious microorganisms’’ (FAO/WHO, 2003). Also, the Exponential model is a non-threshold model, which implies that there is no ‘‘minimum infectious dose’’. A key attribute of the model is its log-linearity (log dose vs. log probability of illness) at low doses; this implies that at low doses, a single serving with a specified level of contamination has the same public health impact as 10 servings

with 10-fold fewer organisms (Giovannini *et al.*, 2004). In this model, D stands for the calculated dose of *L. monocytogenes* per serving, at the moment of consumption. The simple exponential equation (Haas, 1983), was used.

$$P=1-e^{-rxD} \quad \text{Eq.(6)}$$

This is considered a good model for calculation of the probability of acquisition of listeriosis per serving (FAO/WHO, 2004; Giovannini *et a.l*, 2007; Garrido *et al.*, 2010), where *P* stands for the probability of infection at dose (D), D is the dose (CFU) and r is a model parameter specific for each pathogen. Different values of r were considered according to the three sub-populations prior mentioned.

2.3.3. Risk characterization

In this section the probability of illness by eating traditional dry fermented smoked sausage per year was obtained.

2.3.3.1 Consumption data

Consumption data regarding Porto, the major city in the North of Portugal, was obtained through a report elaborated by the Service of Hygiene and Epidemiology, of the Medical School, of Porto University, in collaboration with the Portuguese food safety official authority, ASAE (Lopes *et al.*, 2006). This kind of information regarding the whole Portuguese population is scarce, so these data were extrapolated, by considering the same average value. The total Portuguese population was approximated to 10 million people. Point-estimate values were used to describe these variables. Three population subgroups were considered: intermediate age, elderly over 65 years of age and perinatal exposure.

Data regarding these population subgroups were extracted from the Demographic Statistics Report 2007, from Instituto Nacional de Estatística (INE, 2008).

Finally, the total cases of listeriosis *per annum*, in Portugal, due to traditional dry fermented smoked sausages was calculated multiplying the probability of illness due to one serving by the total number of servings contaminated (total number of servings consumed per year multiplied by the respective % prevalence of *L. monocytogenes*) consumed by the population subgroup (Eq.7).

Predicted nº of listeriosis per year = Size of sub-group(persons) × Nºservings/(year·person) ×Probability of listeriosis/serving ×Simulated *L.m.* prevalence(%) Eq. (7)

The number of listeriosis cases per year was determined at two distinct consumption periods (CPs). The two distinct CPs were a hypothetical period during storage of products as determined by a normal distribution (CP1), and the end of a shelf life of 90 days (CP2) (see Section 2.8.1.4). The flowchart in Fig. 2.4 depicts the calculations performed to calculate the distribution of predicted listeriosis cases per year, in Portugal, due to the consumption of traditional dry and semi-dry fermented smoked sausages.

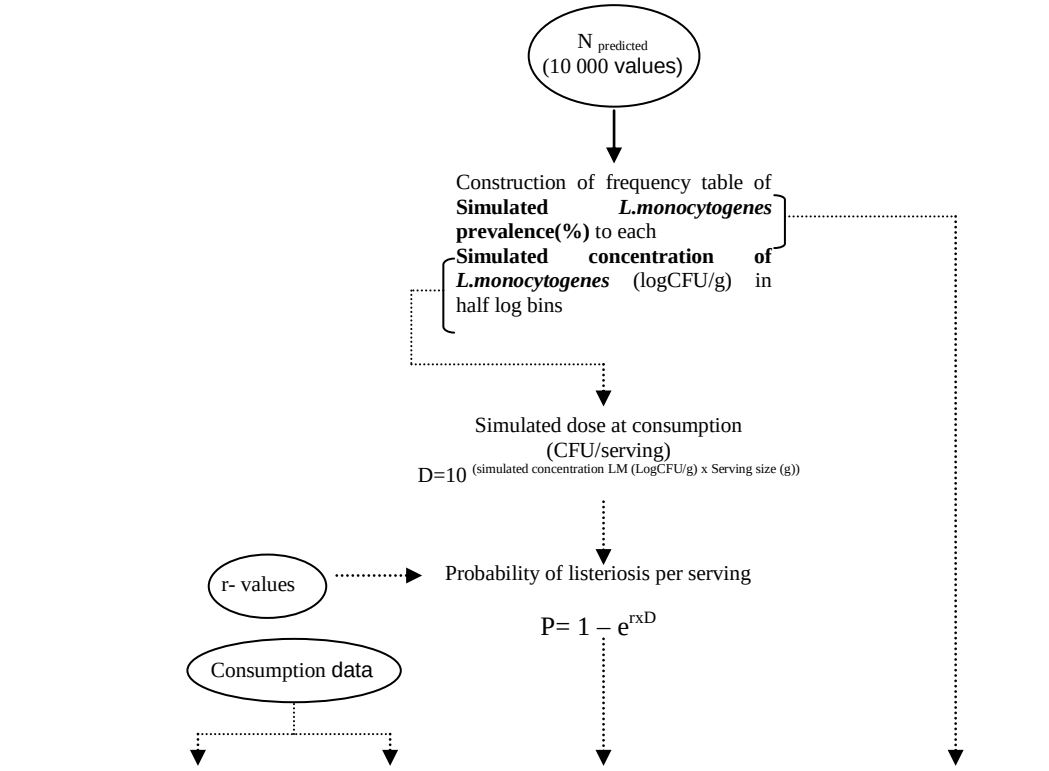


Fig.2.4: Components of the Hazard and Risk Characterization Model.

Using Risk Analysis add-in for Microsoft Excel, @ RISK 4.5, Monte Carlo simulation was used to obtain the effect of variability in the input variables (Poschet *et al.*, 2003, Koutsoumanis and Angelidis, 2007). Each simulation consisted of a random sampling of 100,000 iterations by using the Latin Hypercube sampling method.

2.3.4 Evaluating Uncertainty and Variability

In order to separately assess the uncertainty and variability of the exposure estimates, the approach was separated into two distinct parts:

1. Determining uncertainty and variability of the inputs;
2. Propagating this uncertainty and variability through the model to the output.

In Section 3.3.2, the inputs were characterized either as probability distributions, or by point-estimates (see Table 3.8). In the case of the point-estimate inputs, variability and uncertainty are characterized by the standard deviation around the mean value (Dawber *et al*., 2009). In both ways, they account for variability and uncertainty. As an example, consider a distribution $y=f_1(x)$, characterized by the parameters mean, (m) and standard deviation, (s), that were inferred from a data sample. This distribution, f_1, is simply modelling the variation of the sample as both parameters, m_1 and s_1, are constant values. However, unless the entire population is tested, there is no way to determine the real value of the mean and the real value of the standard deviation. The true value of μ and σ are uncertain, so uncertainty distributions are used for these parameters.

$\mu \sim f_2 (m_2, s_2)$

$\sigma \sim f_3 (m_3, s_3)$

When the distributions specified by the hyperparameters, m_2, m_3 and s_2, s_3, are taken into account, then variability and uncertainty are modelled (Pouillot *et al*., 2007). To obtain the hyperparameters, several techniques are available, including classical statistics, Bayesian inference and bootstrap (OIE, 2004). In this study, hyperparameters were extracted by bootstrapping the data (Frey and Patil, 2002;

Perez-Rodriguez *et al.*, 2006; Dawber *et al.*, 2009; Busschaert *et al.*, 2010). To obtain the hyperparameters of input with distribution f_1 by bootstrapping, it would be necessary to resample the data values, with replacement, and calculate each time the new mean and standard deviation. The distribution f_1 represents variability, but the set of calculated means and standard deviations represents uncertainty about the parameters of this variability distribution, and fit distributions f_2 and f_3.

To propagate the uncertainty and variability through the model to the output, a second-order Monte Carlo simulation (Vose *et al.*, 2001; Pouillot *et al.*, 2003, 2010 a,b, Mokhtari and Frey, 2005; Perez-Rodrigues *et al.*, 2006; Mataragas *et al.*, 2010; Busschaert *et al.*, 2010) was reproduced in an Excel spreadsheet.

The second-order Monte Carlo simulation is performed as follows:

(1) a set of hyperparameters are randomly sampled from their respective distributions; (2) the exposure assessment is performed using 1000 Monte Carlo iterations, taking these hyperparameters as fixed. This exposure assessment takes into account the variability of all parameters conditionally to the hyperparameters, and leads to a distribution of predicted growth of *L.monocytogenes* reflecting the variability of exposure among the population. These values are then used to calculate the predicted number of cases of listeriosis per year, as described in 2.3.2 and 2.3.3.

(3) 100 repetitions of Steps (1) and (2) are performed, leading to 100 distributions of the risk.

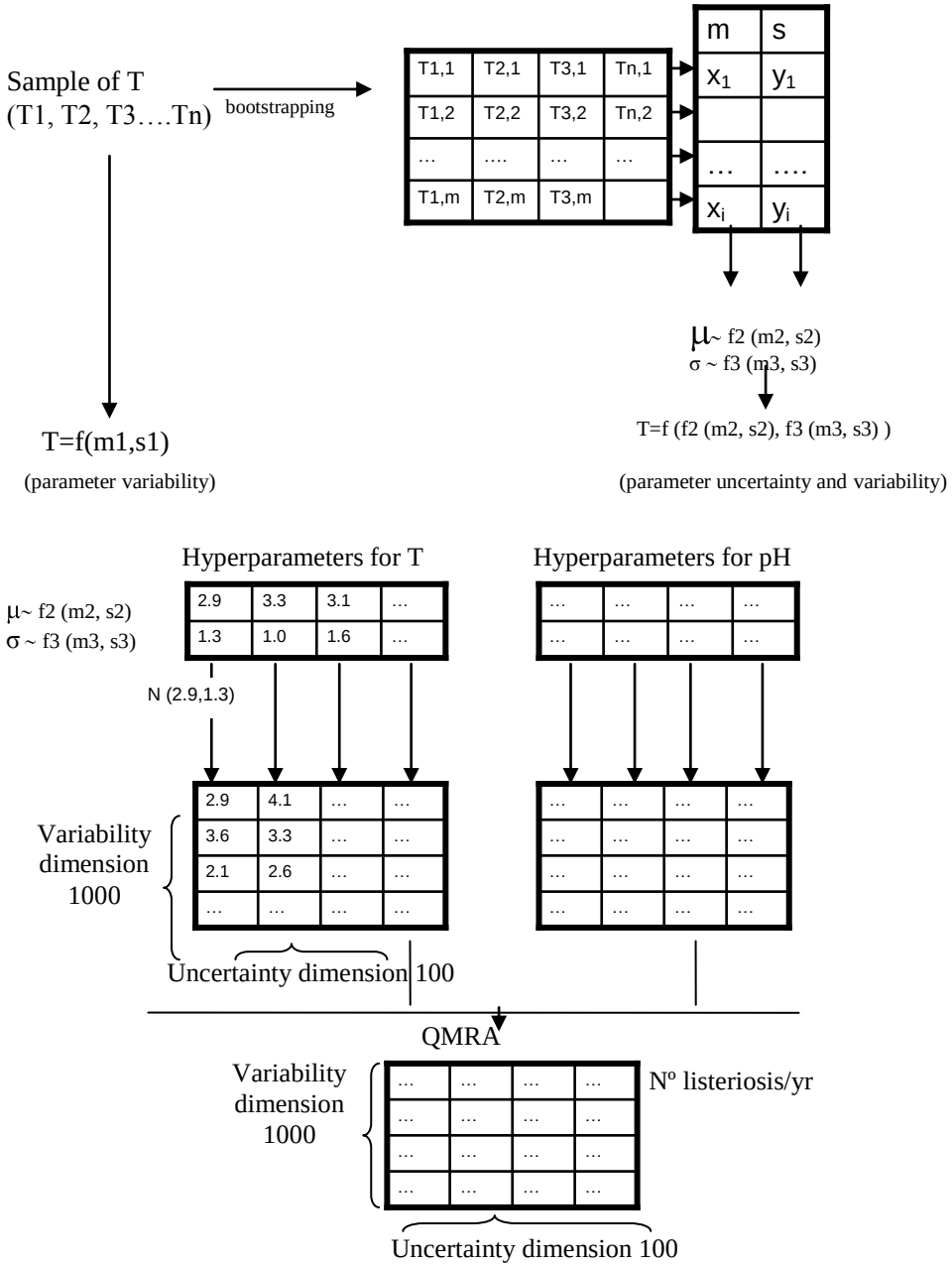


Fig. 2.5 Two dimensional **s**imulation framework (adapted Pouillot, 2007)

(4) The 100 distributions of the predicted number of listeriosis cases (representing the uncertainty) is plotted with the distribution calculated without considering parameter uncertainty (Pouillot *et al.*, 2007), in Section 3.3.4.

2.3.5 Sensitivity analysis of the QMRA

Sensitivity analysis is a way of determining the degree of correlation between the output variables and their associated inputs. Correlation is a quantitative measurement of the relevance of the relationship. Rank order correlation is generally preferred as no assumptions are made about the nature of the relationship (OIE, 2004). With this analysis, correlation coefficients are calculated between the output values and each set of sampled input values. The results of each form of sensitivity analysis can be displayed as a "tornado" type chart, with longer bars at the top representing the most significant input variables. (FAO/WHO, 2009).

2.4 Risk management: Food Safety Objectives (FSO), Process Standards and Process Criteria

In order to comply to the FSO, some risk management actions are explored and its impact on the final risk evaluated (Andersen and Nørrung, 2010).

2.4.1 Establishment of *in-house* "Performance Objectives" to achieve the Food Safety Objective for *L. monocytogenes* in fermented dry meat products

In this case, the "Performance Objectives" proposed are to assure that the concentration of *L. monocytogenes* shall not exceed 10 CFU/g and 1 CFU/g, at CP1, at expedition.

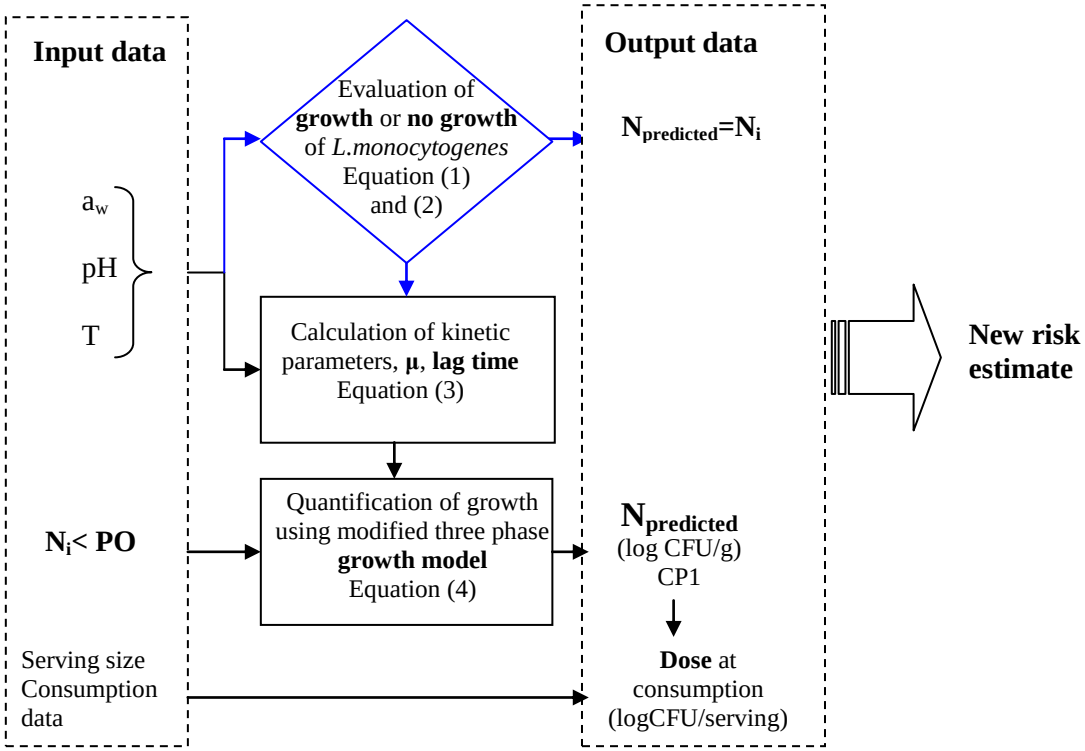


Fig.2.6 New Exposure assessment model, considering "Performance Objective" limiting the Final Product contamination with *L. monocytogenes*

PO_1: $N_{predicted}$<10 CFU/g

PO_2: $N_{predicted}$<1 CFU/g

In practical terms, the values of the simulation of $N_{predicted}$, the contamination level of the product at the exit of the processing plant, are not allowed to exceed the "Performance Objective" in question. In @Risk, the input distribution is truncated at 1 CFU/g and 10 CFU/g. This scenario assumes an improvement of hygiene standards of the facilities and process (Gounadaki *et al.*, 2008).

2.4.2 Establishment of "in-house" Performance Criteria to achieve the Performance Objective previously proposed.

2.4.2.1. Non-thermal inactivation as a possible Process Criterion

Bacteriocins are biocontrol agents that minimize or obviate the use of preservatives, attaining a similar protective effect towards pathogens, maintaining the traditional characteristics of the product, in a more "natural" way, as perceived by the consumer. Most studies on the *in situ* effect of bacteriocins against *L. monocytogenes* in meat and meat products have been carried out using bacteriocinogenic strains of *Lactobacillus* (Benkerroum, 2003), *Pediococcus* (Albano *et al*., 2007a), and *Brochothrix thermosphacta* (Castellano *et al.* , 2008). These have been used in products such as sausage merguez (Benkerroum *et al*., 2003), in the ripening of Sardinian sausage (Greco *et al.*, 2005) and in the smoking of "alheiras" (Albano *et al.*, 2009),

The model developed in Section 3.4.2.11 is applicable to the smoking process, which was not included in the exposure assessment model. But the output of the smoking process, regarding the contamination of the final product is indeed one of the inputs of the exposure assessment model. Lactic Acid Bacteria protective culture may be added to the batter, before stuffing and smoking (ripening) process. To assess the effect of this strategy, it is necessary to infer about the contamination of the batter, so a reverse calculation was made, using the fitted distribution for the initial contamination of the final product, at the processing plant endpoint, and using the model developed of the effect of the smoking

process to calculate an hypothetical distribution of contamination of *L. monocytogenes* in the batter, as described in Fig. 2.7.

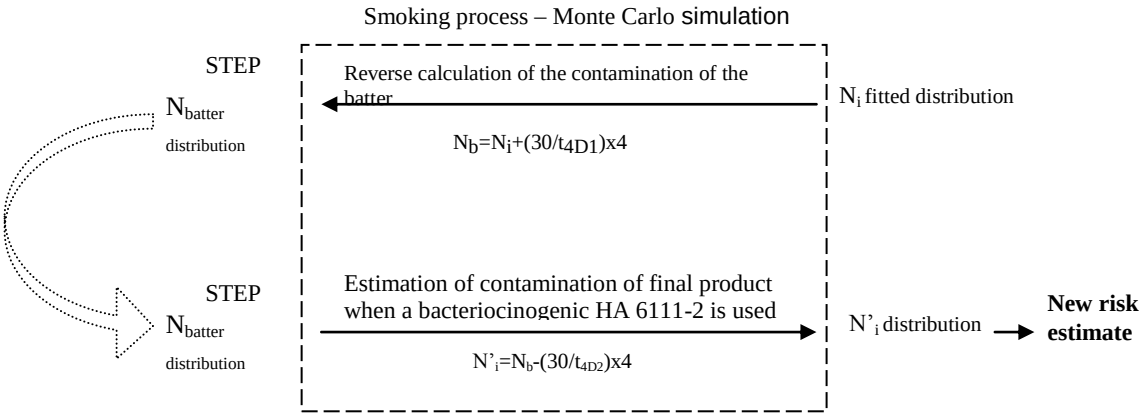


Fig. 2.7: Schematic representation of calculation of a possible Process Criterion as a risk management action.

A Monte-Carlo simulation with 10,000 iterations was used. Now, it is possible to apply the model that describes the effect of the smoking process plus the activity of the bacteriocinogenic HA 6111-2 and, through a stochastic simulation as before, calculate a new distribution of contamination of *L. monocytogenes* and a new estimate of risk.

2.4.2.2. Water activity of final product below 0.92 and addition of bacteriocinogenic HA 6111-2 as a possible Process Criterion

Hurdle technology, derived from the understanding of the hurdle effect (Leistner, 2000), refers to the deliberate combination of existing characteristics such as water activity, in this case, and novel preservation techniques such as the use of bacteriocins, in order to establish a series of more relatively mild, selective

preservative factors (hurdles) that spoilage and pathogenic microorganisms should not be able to overcome (Castellano *et al*., 2008). In this scenario, both the control of a_w of final product and the addition of bacteriocinogenic strain (Fig. 2.8), comprise the hurdle in order to achieve the desired risk level.

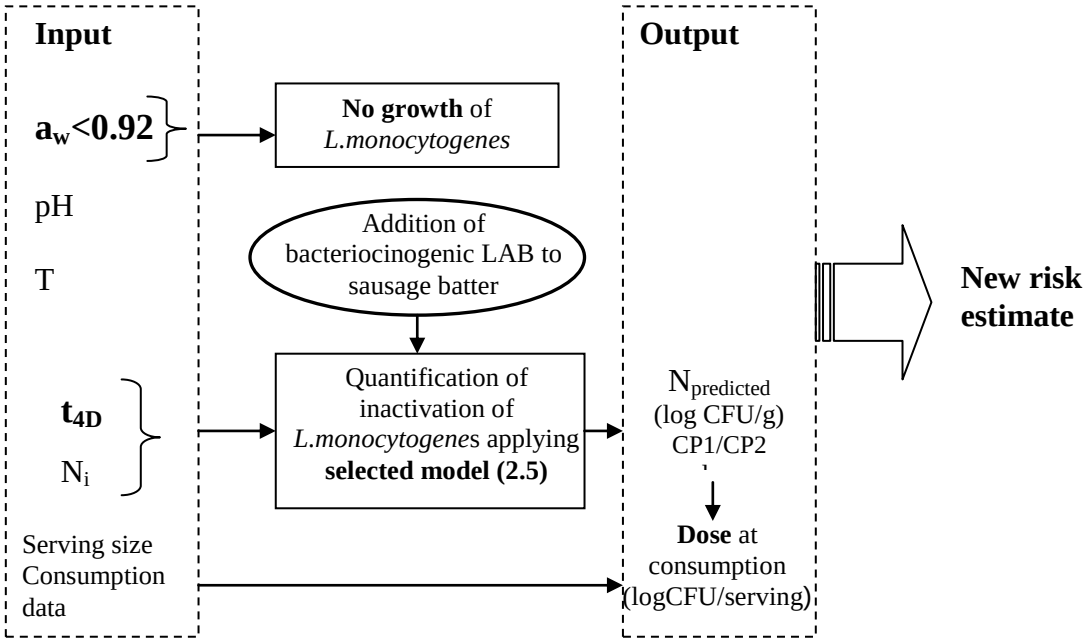


Fig.2.8 New exposure assessment model, considering a_w<0.92 and the use of a bacteriocinogenic LAB.

2.4.2.3 Screening for bacteriocin - producing lactic acid bacteria

Tryptone soya broth with yeast extract (TSB + YE, LabM) agar plates were evenly spread with each of the target pathogen and drops (10 μL) of LAB cultures, grown in MRS/M17 broth at 30 ºC for 24 h, were spotted on the lawns of pathogens and incubated overnight at 30 ºC. *Lactobacillus sakei* CTC 494 (IRTA Meat Technology Centre Collection Monells, Spain) was used as an anti-listerial reference strain. *Listeria innocua* PHLS 2030c (from Central Public Health

Laboratory, Colindale, London) and *L. monocytogenes* 54 (from Escola Superior de Biotecnologia, UCP, Porto, Portugal); *St. aureus* ATCC 29213 (American Type Culture Collection), *E. coli* NCTC 9001 (National Collection of Type Cultures, Central Public Laboratory Service, London, UK), *E. coli* 0157: H7 (ESB), *Ec. faecalis* ATCC 29212, *Salm. Typhimurium* (ESB) and *Salm. Enteriditis* NCTC 05188 were used as target bacteria for the inhibitory effects of LAB. Inhibition was recorded as positive if a translucent halo zone (i.e. no or restricted growth) was observed around the spot. For the positive strains, characterization of the antimicrobial activity was performed according to Tomé *et al*., (2006). Culture broths were centrifuged (Rotina 35R, Hettich, Germany) at 3382 ×g for 15 min, at 4 ºC. The clear supernatants were sterilized by membrane filtration (0.2 μm, Corning Incorporated, Corning 431220, Germany). The pH of the cell-free supernatants was adjusted to 6.5 with NaOH (1 N) and then aliquots treated with catalase (Sigma-Aldrich; 500 IU/mL, sterile) and trypsin (Sigma-Aldrich; 0.1 mg/mL, sterile), for 1 h at 37 ºC. Cell-free supernatant, neutralized cell-free supernatant treated with catalase and neutralized cell-free supernatant treated with catalase and trypsin, were spotted against the target organisms.

2.4.2.4 Identification and characterization of putatively bac+ LAB

2.4.2.4.1 Phenotypic and biochemical characterization

Isolates were streaked onto the respective agar growth media and propagated twice in MRS or M17 broth at 30 ºC for 24 h before study. Cell morphology was observed using an optical light microscope. All isolates were tested for CO_2

production from glucose in MRS agar adjusted to pH 7.0, fitted with Durham tubes (Samelis and Metaxopoulos., 1999; Mackey *et al.*, 1993, Drosinos *et al.*, 2005). Incubation was for 48 h at 30ºC. Isolates were considered heterofermentative if gas was formed. Growth at 10 ºC and 45 ºC was tested by incubating the isolates in MRS or M17 broth (pH 7.0) for 7 and 2 days, respectively. Growth was recorded by an increase in turbidity. As all isolates grew well in MRS, the following tests were performed in this medium, to simplify procedures. Hydrolysis of esculin was tested by adding 2 g/L esculin (Sigma-Aldrich) and 5 g/L ferric ammonium citrate (Sigma-Aldrich) to the basal medium (MRS without glucose and meat extract and with 0.16 g/L bromocresol purple, pH 7.0). Incubation was as described before. A black colouration of the medium was regarded as a positive reaction. Growth in the presence of 6.5% (w/v) NaCl, and at pH 4.0 and pH 9.6 was determined in MRS broth, adjusted with 1 N HCl or NaOH before autoclaving and supplemented with bromocresol purple. All tests were performed in triplicate.

2.4.2.4.2 Genotypic characterization

DNA extraction

The method used was described by Destro *et al*., (1996) and optimized in ESB Food Microbiology laboratory for some Gram-positive bacteria, including enterococci. One colony of each presumptive bacteriocinogenic isolate, grown overnight at 37 ºC on BHI agar (Difco, Heidelberg, Germany), was inoculated into 3mL of BHI broth (Difco). After overnight incubation at 37 ºC, 1 mL of each

cellular suspension was transferred to an eppendorf tube and centrifuged at 8000 ×g at 4 ºC (Rotina 35R) for 5 minutes. The supernatant was discarded and the pelleted cells were re-suspended in 1mL of saline water (0.9% w/v) and centrifuged at 8000 ×g for 5 minutes, twice. The final cells were re-suspended in eppendorf tubes containing 50 μL of sterile distilled water and then placed in boiling water for 15 minutes. Five microliters of the boiled suspension was mixed with 995 μL of water in a quartz spectrophotometer cuvette and the absorbance at 260 nm read in a spectrophotometer (Bio-Rad). This suspension was diluted with water in order to obtain a 200 μL solution with an absorbance of 1.8.

DNA quantification

In a *quartz cuvette*, with 1 cm path length, 10 μL of the extracted solution and 490 μL of water were mixed and the absorbance at 260 nm was read in a spectrophotometer (Bio-Rad). DNA concentration was calculated according to the formula:

$$[\text{DNA}]\ (\text{ng}/\mu\text{L}) = \frac{\text{Abs 260 nm x 50 x V total}}{\text{V DNA}}$$

V total – volume of extracted solution (10 μL) and water (490 μL) in the quartz cuvette

V DNA – volume of DNA in the quartz cuvette (10 μL)

The purity of samples was estimated by reading the absorbance at 260 nm and 280 nm and calculated the ratio Abs_{260}/Abs_{280}. A value lower than 1 indicates that the protein quantity is lower than the nucleic acid quantity.

2.4.2.4.2.2 Genus-specific PCR

A PCR assay for the identification of the genus *Enterococcus* was carried out. *Ec. faecalis* ATCC 29212 was used as the positive control and the primers used were EntF (MWG Biotech AG) (5' – TACTGACAAACCATTCATGATG – 3') and EntR (MWG Biotech AG, Ebersberg) (5' – AACTTCGTCACCAACGCGAAC – 3'), according to the method described by Ke *et al*. (1999). The expected product size was 112 bp.

PCR conditions

PCR amplifications were performed in a ThermoCycler (Bio-Rad) in 0.2 mL reaction tubes each with 20 μL of mixtures using 0.20 mM of each primer (EntF and EntR), 200 μM of dNTP's (TaKara Ex Taq™, TAKARA BIO INC., Otsu, Shiga, Japan), 1X of PCR Buffer (TAKARA BIO INC.), 0.5 mM of $MgCl_2$ (TAKARA BIO INC.), 1U of Taq polymerase (TAKARA BIO INC.) and 100 ng/μL of enterococcal DNA. Amplification reactions were performed under the following conditions: initial cycle of 94ºC for 5 minutes; 35 cycles of 94ºC for 30 seconds, annealing temperature of 50ºC for 30 seconds, 72ºC for 30 seconds; a final extension step of 72ºC for 7 minutes and thereafter cooled to 4ºC. For each PCR reaction a negative control (sample without template) and a positive control (sample with DNA from strain *Ec. faecalis* ATCC 29212) were included.

Gel electrophoresis

All amplification products were combined with 3 μL of loading buffer (Bio-Rad) and 15 μL of these mixtures were applied to a 2.0% (w/v) agarose gel in 1X TAE Buffer (Bio-Rad); (40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH 8.3; Bio-Rad) containing 0.5 μg/mL of ethidium bromide. The electrophoretic separation occurred at 90 V for 2 h. The gels were photographed on a U.V. transilluminator (GelDoc2000, Bio-Rad) and image analysis was accomplished using Quantity One® software (Bio-Rad).

2.4.2.4.3 Antibiotic susceptibility testing

The minimal inhibitory concentrations (MICs; μg/mL) for the presumptive bacteriocinogenic strains were determined by the agar microdilution method, according to National Committee for Clinical Laboratory Standards (NCCLS, 2004). Each test was carried out on Muller-Hinton agar (MHA) (bioMérieux, Marcy l'Etoile, France) with cation adjusted for penicillin G and ampicillin, BHI medium (Difco) for vancomycin and on MHA medium for the other antibiotics investigated, gentamicin, tetracycline, chloramphenicol, ciprofloxacin, rifampicin, gentamicin and nitrofurantoin. *Ec. faecalis* ATCC 29212 and *St. aureus* ATCC 25213 were used as quality control strains. All the antibiotics were purchased from Sigma Chemical Co., with the exceptions of rifampicin and tetracycline, kindly supplied by Labesfal (Tondela, Portugal). The inoculum was prepared from an overnight culture on MRS plates, by suspension in sterile Ringer's solution in order to obtain turbidity equivalent to 0.5 McFarland standards. For each

antibiotic susceptibility determination, at least duplicate experiments were performed.

2.4.2.4.4 Determination of biogenic amine-forming capacity

All presumptive bacteriocinogenic strains were screened for the production of histamine, tyramine, putrescine and cadaverine, according to the method described by Bover-Cid and Holzapfel (1999). Each strain was sub-cultured seven times in MRS broth with 0.1% (w/v) of each precursor amino-acid (all from Sigma-Aldrich); tyrosine free base for tyramine, histidine monohydrochloride for histamine, ornithine monohydrochloride for putrescine and lysine monohydrochloride for cadaverine, and supplemented with 0.005% (w/v) of pyridoxal-5-phosphate (Sigma-Aldrich), in order to promote enzyme induction. Then, the strains were spotted in duplicate on the Bover-Cid medium plates with and without each amino-acid (as control) and incubated at 37 ºC for 4 days under aerobic conditions, in parallel. Positive reactions were confirmed when a purple colour was produced or tyrosine precipitate disappeared from around the colonies.

2.4.2.4.5 Production of gelatinase, lipase and DNase

The production of extracellular enzymes was assessed according to Tiago *et al*., (2004). Briefly, the production of lipases was assayed in modified Luria–Bertani broth (MLB) agar supplemented with 2.0 g/L of $CaCl_2$ and 10 g/L of Tween-80 (Sigma-Aldrich). A positive reaction was indicated by a clear halo around the colonies. The production of gelatinase was assayed in MLB broth supplemented

with 50.0 g/L of gelatin. Tubes inoculated with fresh grown colonies, using a titanium loop, were incubated at 30 ºC for 24 h to 72 h and then placed into the refrigerator for approximately 30 min. If the bacteria did not produce gelatinase the medium remained solid. The presence of sufficient gelatinase liquefied the medium even when placed in the refrigerator.

For DNase testing, a Methyl Green DNAse agar (Difco, Heidelberg, Germany) was used as described by Ben-Omar *et al*. (2004). A clear halo around colonies after incubation of plates at 37 °C for 48 h was considered a positive result. *St. aureus* ATCC 25213 was used as a positive control in all tests.

2.4.2.4.6 Biofilm plate assay

Presumptive bacteriocinogenic strains were tested for production of biofilm using a protocol based on that described by Stepanović *et al*. (2000). Bacteria were grown overnight at 37 °C in MRS broth. Microplates with 96 wells were filled with 180 µL of MRS and 20 µL of overnight culture, and the plates were then incubated at 30 °C for 72 h, for the batch and fed-batch assay. For fed-batch assay, 100 µL of medium were discarded every 24 h and filled with 100 µL of fresh culture medium. After 72 h, the culture medium was then discarded, and the wells were gently washed three times with 200 µL of sterile deionised water without disturbing the biofilm at the bottom of the wells. Then the attached cells were fixed with 250 µL methanol and the plates were dried at room temperature for 15 minutes and stained with 2% Hucker's crystal violet for 5 min. Excess stain was removed by rinsing the plates under tap water. Adherent cells were suspended

with 300 µL of acetic acid (30%) and quantified by measuring the optical density (OD) at 630 nm using a microplate reader (Model 680, Bio-Rad Laboratories). All experiments were done six times for each strain and the results averaged. OD values from the wells that had not been inoculated with bacteria were used as negative controls and two positive controls also have been used: *Ec. faecalis* P1 and *Ec. faecalis* F2 (from a collection of Tracy Eaton).

The cut-off value (ODc) for determining a biofilm producer was defined as two times the mean OD value of the negative control (Yi *et al*., 2004). Strains were classified as: non-biofilm producers (OD ≤ ODc), weak biofilm producers (ODc < OD ≤ 2 x ODc), moderate biofilm producers (2 x ODc < OD ≤ 4 x ODc) and strong biofilm producers (4 x ODc > OD) (Stepanović *et al*., 2000).

2.4.2.4.7. PCR amplification of virulence genes

PCR procedures were performed on total-cell DNA extracted according to the method of Destro *et al*. (1996). The primers used for the amplification of genes *esp, agg, gel*E, *efa*A_{fm} and *efa*A_{fs} were described by Eaton *et al.* (2001) and primers for the cytolysin genes were developed by Semedo *et al*. (2003). All the primers were purchased from MWG Biotech AG. PCR amplifications were performed in a DNA thermal cycler (My Cycler™ Thermal Cycler) in 0.2 mL reaction tubes with mixtures (25 µL each) using 1 × PCR buffer (MBI Fermentas), 2.5 mM $MgCl_2$ (MBI Fermentas), 0.1 mM deoxynucleoside triphosphates (dNTP's) (Abgene, Schwerte, Germany), 0.5 µM of each primer, 2 U of Taq DNA polymerase (MBI Fermentas) and 100 ng of DNA. Amplification reactions were

as follows: initial cycle of 94 ºC for 3 min, 35 cycles of 94 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 2 min, a final extension step of 72 ºC for 7 min and thereafter cooled to 4 ºC. A 5 µL aliquot of the amplification mixture was combined with 3 µL of loading buffer and the preparation was electrophoresed on 1.0 % (w/v) agarose gel at 90 V for 2 h. A 100-bp PCR DNA ladder (Bio-Rad Laboratories) was used as a molecular weight marker. The positive controls used were: *Ec. faecalis* DS 16 (*cyl)* (from the culture collection of C.B. Clewell, Department of Oral Biology, School of Dentistry, University of Michigan, Ann Arbor, USA), *Ec. faecalis* F2 (*agg*), P1 (*efa*A_{fs}), P36 (*gel*E, *esp*) and *Ec. faecium* P11 (*efa*A_{fm}) (from the culture collection of Tracy Eaton, Division of Food Safety Sciences, Institute of Food Research, Norwich, United Kingdom).

2.4.2.4.8. PCR conditions

The method described by Dukta-Malen *et al*., (1995), with some modifications, was followed. PCR amplifications were performed in a ThermoCycler (Bio-Rad) in 0.2 mL reaction tubes each with 25 µL of mixtures using 0.5 mM of each primer, 0.1 mM of dNTP's (AbGene), 1X of PCR Buffer (MBI Fermentas), 2 mM of $MgCl_2$ (MBI Fermentas), 1U of Taq polymerase (MBI Fermentas) and 100 ng/µL of enterococcal DNA.

Amplification reactions were performed under the following conditions: initial cycle of 94 ºC for 2 minutes; 30 cycles of 94 ºC for 1 minute, annealing temperature of 51.6 ºC for 1 minute, 72 ºC for 1 minute; a final extension step of 72 ºC for 10 minutes and thereafter cooled to 4 ºC. For each PCR reaction a negative control (sample without template) and a positive control were included.

2.4.2.4.9. Gel electrophoresis

The electrophoresis occurred at 90 V and was done as described above in section 2.4.2.4.7

2.4.3 Modelling the antilisterial activity of bac+ *Ped. acidilactici* HA-6111-2 during the smoking process

This section describes the experimental setup at pilot scale, of a challenge test to evaluate several factors (smoking process, effect of bacteriocinogenic strain, effect of competition by non-bacteriocinogenic strain of LAB) on the survival of *L. innocua* (as a surrogate for the pathogen *L. monocytogenes*) in pre-inoculated sausages and following microbiological and chemical analyses (2.4.3.3 to 2.4.3.4). *Listeria. innocua* is an adequate surrogate for *L.monocytogenes* (Vaz-Velho *et al.*, 1998), Friedly *et al.*, 2008 and NACMCF, 2009). The data obtained will then be used in the modelling process (2.4.3.5).

2.4.3.1 Sausage preparation, sampling, and inoculation procedure

The seasoned meat batters for "Salpicão'' and "Chouriça", ready to fill, were kindly provided by industrial producer A, transported to the laboratory in portable, insulated cold-boxes and stored at 3 ºC overnight. As described in Results and Discussion – Section 3.6.1.9, the work described henceforward uses the bacteriocin positive *Ped. acidilactici* HA-6111-2 (Albano *et al.*, 2007a, 2007b). The antagonistic effect of the bac+ *Ped. acidilactici* HA-6111-2 strain

against a cocktail of *L. innocua* (*L. innocua* 2030c, *L. innocua* NCTC 11288 and *L. innocua* NCTC 10528) was studied at a pilot plant; a non-bacteriocinogenic *Ped. acidilactici* strain HA-2485-3 (Albano *et al.*, 2007a) was used as a control. The organisms were sub-cultured twice (24 h at 30 ºC) in 10 mL MRS (HA-6111-2 and HA-2485-3) or TSB (*L. innocua*), using a 1% (v/v) inoculum. An aliquot (40 mL) of each bacterial suspension (10^8 CFU/mL for LAB strains and 10^7 CFU/mL for the *L. innocua cocktail*) was added to 4.0 kg of seasoned batter of ‘‘Salpicão’’ and to 4.0 kg of seasoned batter of “Chouriça” placed in disinfected containers, resulting in five different batches, as described below. After ensuring good mixing of the inoculum with the seasoned meat (manually massaging the mix with disposable disinfected gloves) the different batches were manually stuffed into casings. The smoking process (Fig. 2.9) occurred during 4 to 5 weeks (see below point 2.5.2.) and the resulting sausages were then vacuum packed in a Multivac-Gastrovac (Multivac Sepp Haggenmüller KG, A300/41/42, Berlin,Germany). Packs were stored for 90 days at room temperature and analysed (regarding *Listeria* spp., LAB, pH and $a_{w;}$ see below in points 2.5.3, 2.5.4.) immediately after stuffing (week 0) and during smoking at week 1, 2, 3, 4 and 5 and after 90 days of storage time, at room temperature, according to the manufacturer’s label instructions. The experimental conditions were: uninoculated “Salpicão” and “Chouriça” as controls, (LI) “Salpicão” and “Chouriça” inoculated with a cocktail of *L. innocua*, (LABpos) “Salpicão” and “Chouriça” inoculated with *Ped. acidilactici* HA-6111-2 (bacteriocinogenic strain), (LI+LABpos) “Salpicão” and “Chouriça” inoculated with cocktail of *L. innocua* and *Ped.*

acidilactici HA-6111-2, (LI+LABneg) "Salpicão" and "Chouriça" inoculated with cocktail of *L. innocua* and *Ped. acidilactici* HA-2485-3 (non-bacteriocinogenic strain). Two independent experiments were performed.

2.4.3.2**.** Smoking equipment and operational conditions

The smoking process requires low temperatures. For this reason the smoking chamber (AGK, Type 135/12, Wallersdorf, Germany) operated at room temperature (between 4 ºC and 14 ºC), during December 2007-February 2008. Additionally, an extension of the fume pipe from the smoke generator was used in order to cool down the smoke before entry into the smoking chamber. During the drying-smoking process an induced draught was necessary to decrease the humidity in the smoker chamber and moisture in the product. The smoke was produced by the smouldering of wood chips of oak. Sausages were rotated inside the smoking chamber to minimize differences in drying rate, since the ones closer to the inlet damper lost moisture faster. The opening of the inlet damper of the smoker chamber was controlled during smoke generation and drying, to mimic the real process as closely as possible, until the smoking process was concluded.

Fig 2.9. Smoking process of "Salpicão" and "Chouriças" at pilot scale.

2.4.3.3. Microbiological analyses

Twenty-five gram samples were added to 225 mL of sterile buffered peptone water, and homogenized in a stomacher for 2 min. Appropriate decimal dilutions were prepared in Ringer's solution for microbial enumeration: LAB on MRS agar incubated at 30 ºC for 72 h, and on M17 agar also incubated at 30 ºC for 72 h; Enumeration of *Listeria* spp. was performed on PALCAM Agar (Merck) medium and incubated at 30 ºC for 72 h.

2.4.3.4. Chemical analyses

2.4.3.4.1 pH determination

pH was determined as described in section 2.2.1.

2.4.3.4.2 Water activity

The water activity was measured with a calibrated electric hygrometer, Hygroplam AW1 (Rotronic Instrument Corporation, U.S.A.), according to the manufacturer's instructions.

2.4.3.5. Mathematical models and regression software used

To select the best model, the approach described by Besten *et al.* (2006) and Drosinos *et al.* (2006) was adopted.

2.4.3.5.1 Linear model (Van Gerwen, 1998)

The linear model (first order model) is $N_t = N_0 - (\frac{t}{D})$ where N_t is the cell number (log CFU/g) at time *t*, N_0 is the initial population (log CFU/g), *t* is the time (days), and *D* is the time needed for a 1-log reduction of the *L. innocua* population (days). In this process that takes 4 to 5 weeks, the D value is calculated in days, for easier interpretation.

2.4.3.5..2 Log linear model with shoulder (Geeraerd, *et al.* 2000)

Not all inactivation processes always follow first order inactivation kinetics, so non-log linear modelling equations were used.

$$\log 10(N) = \log 10\left[N(0) \cdot e^{-k_{\max} t} \cdot \left(\frac{e^{k_{\max} S_1}}{1 + (e^{k_{\max} S_1} - 1) \cdot e^{-k_{\max} t}} \right) \right]$$

2.4.3.5.3 Log linear model with tail (Geeraerd *et al.*, 2000)

$$\log 10(N) = \log 10\left[(N(0) - N_{res}) \cdot e^{-k_{max}t} + N_{res}\right]$$

It is important to remark that, for this model, tailing is considered for a population remaining constant in time or, otherwise stated, not undergoing any significant subsequent inactivation (Geeraerd, 2005).

2.4.3.5.4 Weibull model (Van Gerwen *et al.*, 1998)

The Weibull model is $N_t = N_0 - (\frac{t}{D})^{\beta}$ where β is an adjustment parameter. *ß* values of <1 correspond to concave upward survival curves, *ß* values of >1 correspond to concave downward curves, and a *ß* value of 1 corresponds to a straight line.

2.4.3.5.5 Albert and Mafart model (Albert and Mafart *et al.*, 2003, 2005)

The model can be written as follows:

$$\log 10(N) = \log 10\left[(N(0) - N_{res}) \cdot 10^{\left(-\left(\frac{t}{\delta}\right)^{p}\right)} + N_{res}\right]$$

This model is able to describe concave, convex or linear curves followed by a tailing effect.

2.4.3.5.6 Biphasic model

Cerf (1977) proposed a two-fraction model, which assumes the existence of two populations, which can be formulated as follows

$$\log 10(N) = \log 10(N(0)) + \log 10(f \cdot e^{-k_{max1}t} + (1-f) \cdot e^{-k_{max2}t})$$

Herein, *f* is the fraction of the initial population in a major subpopulation, (1-*f*) is the fraction of the initial population in a minor subpopulation (which is more resistant than the previous one), and k_{max1} and k_{max2} [1/time unit] are the specific inactivation rates of the two populations, respectively.

2.4.3.5.7 Baranyi model

The Baranyi model is

$$N_t = N_0 + (k \times A_t) - \ln\left[1 + \frac{\exp(k \times A_{t)} - 1}{\exp(N_f - N_{0)}}\right]$$

where N_t is the bacterial population at time *t* (logCFU/g), N_f and N_0 are the maximum and initial populations, respectively (logCFU/g), *k* is the inactivation rate (logCFU/g per day), and *At* is an adjustment function. (Baranyi and Roberts, 1994), which is considered to describe the physiological state of the cells, which defines the lag phase (shoulder period, *ts*).

2.4.3.5.8 Biphasic logistic model

The biphasic logistic model (Whiting, 1993) assumes the existence of a primary population and a secondary population, with different resistencies and aims to take into account a shoulder for both populations.

$$N_t = N_0 + \log\left\{\frac{f \times [1 + \exp(-k_1 \times t_s)]}{1 + \exp[k_1 \times (t - t_s)]} + \frac{(1 - f) \times [1 + \exp(-k_2 \times t_s)]}{1 + \exp[k_2 \times (t - t_s)]}\right\}$$

where *f* is the fraction of initial population in the major population, 1-*f* is the fraction of population in the subpopulation, and k_1 and k_2 are the inactivation rates

of the major population and subpopulation, respectively (log CFU/g per day). When no shoulder is present, then *ts* = 0 and if one population is present (no subpopulation), then *f* = 1.

2.4.3.5.9 Reparametrized Gompertz

The re-parameterized Gompertz model (Zwietering *et al.*, 1990) is:

$$N_t = N_0 - A\exp\left\{-\exp\left[\left(\frac{k \times e}{A}\right)(t_s - t) + 1\right]\right\}$$

where A is the difference between the initial and final populations (N_0 - N_f) (log CFU/g), k is the inactivation rate (logCFU/g per day), t_s is the shoulder period (days), and e is 2.7182.

All inactivation models except the biphasic logistic model and the Baranyi model were fitted to the experimental data using the GynaFit freeware add-in for Microsoft Excel. For the biphasic logistic model, the non-linear regression was calculated using the program Tablecurve2D v2.0 (Jandel Scientific, San Rafael, USA) while for the Baranyi function, the DMFit program was used (Institute of Food Research, Norwich, UK).

2.4.3.5.10 Statistical comparisons and model selection.

R^2- the coefficient of determination which equals 1-SSE/SSTO, with SSE, the sum of Squared Errors, obtained by summing the squared differences between the experimental data and the predicted values, both in log 10-scale, and SSTO the sum of the squared differences between the measured values and the mean of

these measured values. R^2 values close to 1 indicate that the curve comes close to the data.

A_f – The accuracy factor translates how close the predicted values are to observed values. The closest the A_f factor is to 1, the better is the prediction, and can be calculated by:

$$A_f = \exp\left\{\log\left[\sum_{i=1}^{n}\left|\log(x_i^p / x_i^0 \Big/ n\right|\right]\right\}$$

MSE - the Mean Sum of Squared Errors, which can be derived by dividing SSE by the number of degrees of freedom n-k, i.e., the number of data points n minus the number of degrees of freedom k (parameters and initial values) used.

F - The *F* ratio can be used to evaluate the adequacy of a model for describing the data using the lack of fit (LOF) statistic. The residual mean square errors of the model (MSE_{model}) and data (MSE_{data}, measuring error) are compared by using the *F* ratio, according to the method of Zwietering *et al.* (1994):

$$F = \frac{MSE_{model}}{MSE_{data}}$$

LOF gives the deviation of a model from the experimental error. If the deviation of the model is significantly smaller than the experimental error (F ratio< F table value, where f table value is equal to $F_{DF_{data}}^{DF_{model}}$ with α=0.05) then the model is adequate and is accepted. (DFmodel is the total number of data points minus the number of parameters of the model; DFdata is the total number of data points minus the number of time points).

2.4.3.5.11 Estimation of the t_{4D} parameter.

To facilitate the comparison between inactivation curves, the time (in days) needed for a 4-log reduction (t_{4D}) in the *L. monocytogenes* population was the parameter used (Whiting, 1993, Drosinos *et al.*, 2006). For linear inactivation, the following equation is used:

$$t_{4D} = t_s + 4 \times D$$

For non-linear inactivation, Whiting (1993) proposed the following equation:

$$t_{4D} = \frac{\log\left[\frac{1+\exp(-k \times t_s)}{0.0001} - 1\right] + k \times t_s}{k}$$

where *ts* is the shoulder period (days), *D* is the time needed for a 1-log reduction in the *L. monocytogenes* population (days), and *k* is the inactivation rate (per day).

A one-way analysis of variance (ANOVA) comparing the t_{4D} parameters among the different experimental trials was performed to determine whether there was a significant influence of the use of a bacteriocinogenic strain on *L. innocua* inactivation. A two-way ANOVA also was performed to compare the t_{4D} parameters between "Salpicão" and "Chouriça" and determine any potential significant effect of different product size or preparation. Statistical analysis was performed using Excel 3.0.

The resulting value of t4D of treatment LI and LABpos will be used in the new exposure assessment performed when the use of a bioprotective culture as a Process Criterion is studied.

2.4.4. Water activity of final product below 0.92 as a possible Process Criterion

Another strategy is to control the smoking process (Hwang *et al.*, 2009), during which drying of the product occurs, until the products´ characteristics are such that prevent the growth of the pathogen. If the water activity is below 0.92 such goal is achievable, without hindering the organoleptic characteristics of the product. In this scenario, the contamination of the final product, the hygienic conditions in which it is processed, are of utmost importance in the calculation of the new risk estimate (Fig. 2.10).

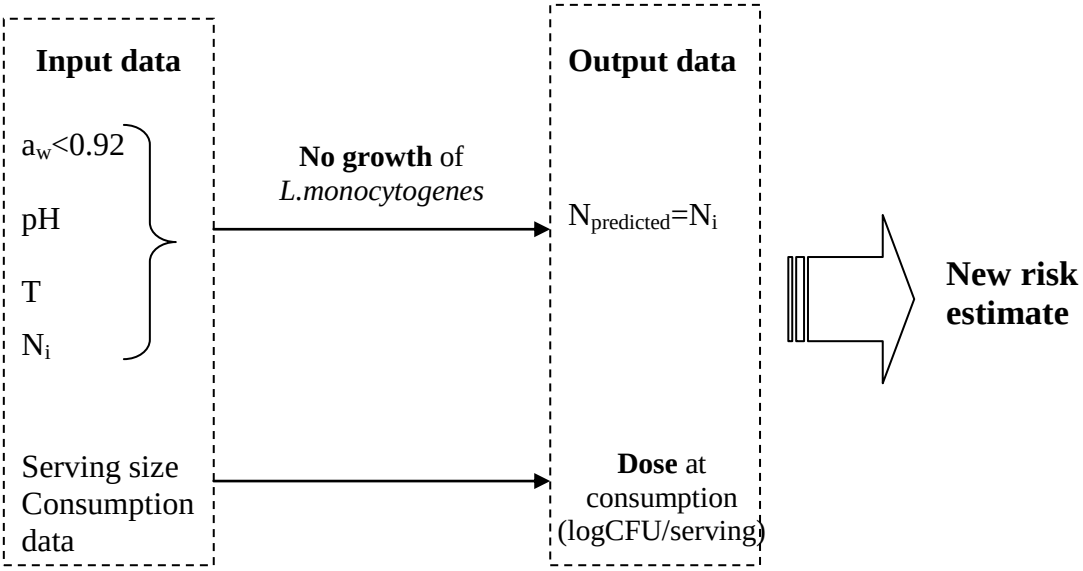


Fig.2.10 New exposure assessment model, considering a_w<0.92

CHAPTER 3. RESULTS AND DISCUSSION

In this section, all the results obtained in this study will be presented and discussed. Sub-sections 3.1 and 3.2 describe the microbiological and chemical characterization pertinent to the determination of the relevant food safety hazards in dry fermented meat products under study. This data will be used in sub-section 3.3, in the construction of a quantitative microbiological risk assessment of the pathogen *L. monocytogenes* in traditional dry fermented smoked sausage from the North of Portugal, "Salpicão" and "Chouriça" de Vinhais. In sub-section 3.4, some risk management scenarios are explored, to reduce the predicted risk. The reduction of the microbial load, as a Performance Criteria and the use of a bioprotective measure were considered in new risk estimations. For the latter, the process of isolation and study of a potential bacteriocinogenic culture is described. A mathematical model was developed to describe the effect of the bioprotective culture and integrate it into the new risk assessment model.

3.1 Microbiological characterization of "Salpicão de Vinhais" and "Chouriça de Vinhais"

3.1.1 Pathogenic bacteria/indicator organisms in final product

In 57 samples of both "Salpicão" and "Chouriça" *St. aureus*, spores of sulphite-reducing clostridia, *E. coli* 0157:H7 and *Salmonella* spp. were not detected in any of the samples. *Listeria. monocytogenes* was considered the pathogen of concern,

being present in 15.8% of the 57 samples, evenly distributed between samples of "Salpicão" and "Chouriça". The results will be presented globally, for "Salpicão" and "Chouriça". The presumption that "Salpicão" and "Chouriça" behave the same way and therefore may be considered as a single class product will be sustained during this study.

20 samples from retail stores were purchased and analysed at a later moment, for the presence of *L. monocytogenes* and *Yersinia* spp., as this is a pork derivative. As it was already established that *L. monocytogenes* was a relevant hazard for these products, it was decided to focus the research on this microorganism. The detection of *Yersinia* spp. was only performed on this set of samples because the analytical method was, only then, fully implemented. *Yersinia* spp. was not detected in this set of samples (no characteristic colonies observed) although 10% of the analysed samples tested positive for *L. monocytogenes*. *Yersinia enterocolitica* has been detected in Turkish dry fermented sausage Sucuk (Asplund *et al*., 1993, Ceylan *et al.*, 2000). Overall, in the 77 samples, 14.3% tested positive for *L. monocytogenes*. In all the samples of ''Salpicão" and ''Chouriça'', no *Salmonella* spp., *E. coli* O157 or *C. perfringens* were detected. *L. monocytogenes* was detected only in three samples of ''Chouriça''. In a previous study (Ferreira *et al.*, 2007a), *E. coli* was detected in concentrations higher than 100 CFU/g. *St. aureus* was also recovered, but in a concentration lower than 10^4 CFU/g. In "alheiras", traditional fermented sausages from the north of Portugal, often produced in the same facilities as "Salpicão de Vinhais" and "Chouriça de Vinhais", thirty-eight lots of "alheira," from 17 producers were analysed;

Campylobacter spp. and *Escherichia coli* O157 were not detected in any sample, and *C. perfringens,* when present, was not at levels of concern with reference to public health; *Salmonella* spp. was detected in two lots of industrially produced "alheiras", and more than 60% of the lots analysed were contaminated with *L. monocytogenes* in concentrations higher than 100 CFU/g (Ferreira *et al*., 2006). In another survey, different results were obtained. *St. aureus*, *C. perfringens* and *Salmonella* spp. were prevalent in 50, 25 and 12.5% of the samples of "alheiras", respectively while *L. monocytogenes*, *Bacillus cereus* and *Y. enterocolitica* were present in smaller numbers and *E. coli*, O157:H7 and *Campylobacter* spp. were not detected (Esteves *et al.*, 2008). *Salmonella* spp (Escartin *et al.*, 1999) and verocytotoxigenic *E. coli* (Villani *et al.*, 2005) were detected in fresh pork sausages. Siriken (2006) detected *Salmonella* spp. and *Listeria* spp. were detected in 7% and 9% of the samples of Turkish sausage soudjouk, while *no E. coli* O157:H7 was detected. In an Italian national survey of cacciatore salami, *Listeria monocytogenes* was recovered in 22.7% of the 1020 samples tested (Gianfranceschi *et al.*, 2009).

The 2003 Risk Assessment for RTE foods by FDA indicates that Dry/Semi-Dry Fermented Sausages were, among others, most likely to be contaminated; although some servings of all food categories were likely to be contaminated at the retail level (FDA/CSFAN, 2003). Prevalence of *L. monocytogenes* in French dry fermented sausages (Thévenot *et al.*, 2005b) at the end of production process was 10%, with contamination levels below 100 CFU/g. *L. monocytogenes* was detected in 11.6% of Turkish fermented sausage "sucuk" (Colak *et al.*, 2007).

Cabedo *et al.* (2008) reported a prevalence of *L. monocytogenes* in several RTE food samples in Catalonia, Spain, from 1.3% to 20%. In a study performed on RTE foods (sausages and other meat products) marketed in Italy, the prevalence of *L. monocytogenes* was 9.5%, although the level of the pathogen in the positive samples was below 10 CFU/g in 94.7% of the cases (Meloni *et al.*, 2009).

3.1.2 Pathogenic bacteria/indicator organisms during the smoking process

The smoking process is a complex system during which several phenomena occur. During this process, there is microbial growth, fermentation (also addressed as ripening or maturation), production of enzymes and organic acids, decrease in a_w and pH changes (Aquilanti *et al.*, 2007; Benito *et al.*, 2007; Esteves *et al.*, 2008; Ferreira *et al.*, 2007a). This affects the evolution of the microflora present during this process.

The results for the microbiological characterization of "Salpicão" and "Chouriça" are summarized in Tables 3.1 and 3.2, respectively.

According to the Commission Regulation (EC) No 2073/2005 (EC, 2007) and the Food Safety Authority of Ireland Guidelines (Anonymous, 2001), "Chouriça" from producer A would be classified as Unsatisfactory as *Enterobacteriaceae* counts were higher than log 4 CFU/g. These organisms were present at high levels in all processing stages. These results are higher than the ones reported by Comi *et al.* (2005), with values ranging from log 3 log CFU/g to log 4 CFU/g.

Table 3.1: Microbiological characterization of "Salpicão"

	Producer	Lot	Seasoning	t (weeks) 0	1	2	3	4	5
Aerobic mesophilic plate count (Log CFU/g)	**A**	**1**	5.2±0.2	6.6 ±0.4	6.6 ±0.0	7.0±0.1	7.37 ±0.16	7.4 ±0.2	>7.5±0.0
		2	5.6±0.2	>7.5±0.00	>7.5±0.0	>7.5±0.0	>7.5±0.0	>7.5±0.0	>7.5±0.0
	B	**1**	n.a.	>7.5±0.00	>7.5±0.0	>7.5±0.0	>7.5±0.0	>7.5±0.0	---
		2	5.9 ±0.1	5.4±0.3	7.3±0.3	>7.5±0.0	>7.5±0.0	>7.5±0.0	---
***Enterobacteriaceae* (Log CFU/g)**	**A**	**1**	4.3 ±0.2	4.7±0.1	4.2±0.1	5.1±0.1	4.2±1.1	4.4 ±0.3	3.5±0.3
		2	4.7 ±0.3	5.1	3.8±3.0	4.8±0.9	2.4±0.0	3.1±0.5	3.1±1.0
	B	**1**	n.a.	3.1±1.2	1.0±0.0	<1.0±0.0	1.4±0.6	3.3±3.3	---
		2	3.7± 0.2	<1.17	2.5 ± 2.1	1.2	<1.0±0.0	1.0±0.2	<1.0±0.0
***St. aureus* (Log CFU/g)**	**A**	**1**	2.0 ±1.0	<1.0±.0	1.9 ±0.2	<1.0±0.0	<1.0±0.0	<2.2± 0.0	<1.0±0.0
		2	<1.0±0.0	1.6±0.8	<1.0±.0	<1.0±0.0	<1.0±0.0	<1.0±0.0	<1.0±0.0
	B	**1**	n.a.	1.4±0.2	<1.0±0.0	<1.0±0.0	<1.0±0.0	<1.0±0.0	---
		2	<1.0±0.0	<1.0±0.0	1.6 ±0.8	<1.0±0.0	<1.0±0.0	<1.0±0.0	---
L. monocytogenes* VIDAS *(*presence per 25g*)	**A**	**1**	n.a.	(-)/(-)	(-)/(+)	(-)/(-)	(-)/(+)	(-)/(-)	---
		2	n.a.	(-)/(-)	(-)/(-)	(-)/(-)	(-)/(-)	(-)/(-)	(-)/(-)
	B	**1**	n.a.	(+)/(+)	(-)/(+)	(-)/(-)	n.a.	(-)/(-)	---
		2	n.a.	(+)/(+)	(-)/(+)	(+)/(+)	(-)/(-)	(-)/(-)	---
***L. monocytogenes* spp. (MPN/g)**	**A**	**1**	n.a.	<0.2±0.0	<0.2±0.0	<0.2±0.0	<0.2±0.0	<0.2±0.0	<0.2±0.0
		2	n.a.	0.3±0.2	0.3±0.1	0.2±0.0	<0.2±0.0	0.30±0.14	<0.2±0.0
	B	**1**	n.a.	<0.2±0.0	<0.2±0.0	<0.2±0.0	0.7±0.7	<0.2±0.0	---
		2	n.a.	0.5±0.4	1.0±0.9	n.a.	1.9±1.9	2.0±0.4	1.7±2.2

TVC –Total viable counts; All tests performed in duplicate
n.a.- not available A,B- Producers

Table 3.2 : Microbiological characterization of "Chouriça"

	Producer	Lot	Seasoning	t (weeks) 0	1	2	3	4
Aerobic mesophilic plate count (Log CFU/g)	**A**	**1**	5.8 ±0.4	6.7 ± 0.1	7.0 ± 0.3	6.8 ±0.4	6.08	---
		2	5.1± 0.8	7.4±0.1	n.a	>7.5±0.0	>7.5±0.0	>7.5±0.0
	B	**1**	6.4± 1.0	6.7±0.3	>7.5±0.0	>7.5±0.0	>7.5±0.0	---
		2	5.6± 0.4	5.1±0.0	>7.5±0.0	>7.5±0.0	>7.5±0.0	---
***Enterobacteriaceae* (Log CFU/g)**	**A**	**1**	4.3± 0.2	3.5±0.2	4.6±0.0	4.3±0.1	4.18	---
		2	4.9 ±0.1	4.7±0.8	5.5±0.4	4.9±0.3	5.0±0.5	4.9± 0.3
	B	**1**	4.3 ±0.2	3.6±0.0	2.4±1.3	1.6±0.2	1.3±0.5	---
		2	3.8±0.2	2.9± 0.8	1.3 ± 0.0	<1.0±0.0	<1.0±0.0	---
***St. aureus* (Log CFU/g)**	**A**	**1**	<1.0±0.0	<1.0±0.0	1.66 0.25	<1.0±0.0	<1.00	---
		2	<1.0±0.0	<1.0±0.0	<1.0±0.0	<1.0±0.0	<1.0±0.0	<1.0±0.0
	B	**1**	<1.0±0.0	2.0±0.2	<1.0±0.0	<1.0±0.0	1.58±0.83	---
		2	<1.0±0.0	<1.0±0.0	<1.0±0.0	<1.0±0.0	<1.0±0.0	---
***L. monocytogenes VIDAS* (presence per 25g)**	**A**	**1**	n.a.	(+)/(+)	(+)/(+)	(-)/(-)	(+)/(+)	---
		2	n.a.	(-)/(-)	(-)/(-)	(-)/(-)	(-)/(-)	(-)/(-)
	B	**1**	n.a.	(+)/(+)	(+)/(+)	(+)/(+)	(+)/(+)	---
		2	n.a.	(+)/(+)	(+)/(+)	(-)/(-)	(+)/(+)	---
***L. monocytogenes* (MPN/g)**	**A**	**1**	n.a.	<0.2±0.0	<0.2±0.0	0.2±0.0	<0.0±0.0	---
		2	n.a.	3.0±4.0	n.a.	0.3±0.1	0.2±0.0	0.2±0.0
	B	**1**	n.a.	n.a.	13.0±12.7	3.0±0.4	17.5 ± 9.2	---
		2	n.a.	1.1±0.0	28.5±0.2	n.a.	63.2±40.7	---

The high levels observed in final products can be related to the availability of nutrients and with the values of pH and $a_{w,}$ being still compatible with the survival of these organisms, and indicates that they are not eliminated by the processing conditions. On the basis of these results it can be inferred that these products were produced under deficient hygienic conditions (Chevallier *et al.*, 2006; Talon *et al.*, 2007; Gounadaki *et al.*, 2008) and/or using raw material of poor microbiological quality (Chevallier *et al.*, 2006; Latorre-Moratalla *et al.*, 2010). *Staphylococcus aureus* were present in samples from both producers A and B at levels not considered hazardous (lower than 4 log CFU/g) but still unsatisfactory (higher than 2 log CFU/g). *Listeria monocytogenes* was detected in both products, and from both producers. Sulphite reducing *Clostridium* spores were not found in any sample, from both producers. The high numbers of aerobic plate counts (APC) in every sample are due, significantly, to the fermentation process and LAB development. In the study developed by Comi *et al.* (2006), no *L. monocytogenes*, *Salmonella* spp. nor *St. aureus* were isolated from the raw materials or during the maturation, in North Eastern Italian traditional fermented sausages. It is widely recognized that Good Hygienic Practices are the basis or a vital part of HACCP (FAO/WHO, 2004). Therefore good manufacturing procedures in processing facilities together with selection of raw materials are of the utmost importance. *Listeria monocytogenes* was detected during the process in both producers, though not in every batch and it seems to be reduced by the smoking process. *Listeria monocytogenes* is frequently found in raw materials and food processing environments, therefore being frequently isolated during the early stages of

sausages fermentations (Talon *et al.*, 2007; Gounadaki *et al.*, 2008). The reduction of this contaminant during the smoking process has been reported in other studies (Thévenot *et al.*, 2005a,b; Drosinos *et al.* 2005; Zdolec *et al.*, 2008; Dourou *et al.*, 2009). This could be the result of a set of hurdles such as low pH, low a_w, high salt concentration, competition with other organisms or the presence of antilisterial compounds. However, Campelos *et al*., (2008) demonstrated that traditional fermented meat sausages from the North of Portugal are highly variable in characteristics such as pH and a_w and some combinations allow the growth of *L. monocytogenes*. During the smoking process, when fermentation and ripening occur, it seems that inactivation of *L. monocytogenes* occurs, as it was detected during the process, but not in the final product (Tables 3.1 and 3.2).

Several studies indicate that during similar processes there is a reduction in the numbers of pathogens; Drosinos *et al.* (2005), found that *Listeria* spp. *Enterobacteriaceae*, *Pseudomonas* spp, yeasts and aerobic spore-formers decreased during fermentation and the ripening process and were below the detection limit in the final product; Lindqvist and Lindblad (2009) studied the evolution of *E. coli*, *L. monocytogenes* and *Y. enterocolitica* inoculated in sausage batters and indicated that the inclusion of a maturation period, above refrigeration temperatures before distribution, might increase the safety of these products; in *teewurst*, (Dourou *et al.*, 2009), a traditional sausage of Germanic origin, challenge testing with *E. coli* O157:H7, *L. monocytogenes*, and *S. Typhimurium* indicated that this product does not provide a favourable environment for the survival of these pathogens, inoculated either into or onto the product, perhaps

due to the presence of autochtonous LAB; similar conclusions are presented by Karakolev (2009), who found *L. monocytogenes* in 12 of 140 Bulgarian market samples of "raw smoked" sausages.

Enterococci counts were between 3.5 and 4.5 log CFU/g in most of the products. Strains of this genus are frequently isolated from fermented sausages, especially in high pH products where no competitive starter cultures are used (Hugas *et al.*, 2003). The metabolic activity of enterococci in the fermenting sausage matrix have not been studied in detail, however, they certainly contribute to sausage aromatization by their glycolytic, proteolytic and lipolytic activities (Sarantinopoulos *et al.*, 2001, Comi *et al.*, 2005, Foulquié Moreno *et al.*, 2006, Di Cagno *et al.*, 2008). The presence of enterococci in foods however, is a concern, as many strains possess virulence traits (Franz *et al*., 1999, 2001, 2003; Giraffa *et al.*, 2002, Cocconcelli *et al.*, 2003; De Vuyst *et al*., 2000), though Barbosa *et al.*, (2009) concluded that enterococci isolated from food samples present fewer virulence traits than those isolated from clinical samples.

Regarding the raw materials used by producers A and B, results are summarized in Table 3.3. "Salpicão" and "Chouriça" use very similar raw materials. Pathogens of concern were not detected in any of the analyzed samples (fresh pork meat, paprika, garlic, laurel and natural pork casings). Total viable counts were satisfactory (lower than 4.0 logCFU/g) for fresh meat while *Enterobacteriaceae* levels were acceptable, as they were higher than 2.0 log CFU/g but lower than 3.0 log CFU/g (EC, 2005).

Aerobic plate counts and *Enterobacteriaceae* levels were high in the natural casings in both facilities, indicating that an improvement of the hygienic status of this component would be advisable. Casings presented counts of *Enterobacteriaceae* higher than the recommended value of 100 CFU/g, in both facilties.

Table 3.3: Microbiological characterization of raw materials

Control points	Facility	Aerobic plate count (logCFU/g)	*Enterobacteriaceae* (log CFU/g)	*St. aureus* (log CFU/g)	*L. monocytogenes* VIDAS (1) (presence in 25g)	*L. monocytogenes* (MPN/g)	*Salmonella* spp. VIDAS (1) (in 25g)
Pork meat	A	2.8± 0.0	2.7	<1	(-)/(-)	<20/100	(-)/(-)
Paprika	A	4.1	4.6	<1	(-)/(-)	<20/100	(-)/(-)
	B	5.1	<1	<1	(-)/(-)	<20/100	(-)/(-)
Garlic	A	2.6	2.1	<1	(-)/(-)	<20/100	(-)/(-)
	B	1.9	<1	<1	(-)/(-)	<20/100	(-)/(-)
Laurel	A	2.9	<1	<1	(-)/(-)	<20/100	(-)/(-)
	B	1.50	<1	<1	(-)/(-)	<20/100	(-)/(-)
Salt	A	<1	<1	<1	(-)/(-)	<20/100	(-)/(-)
Casings	A	>7.5	4.1	<1	(-)/(-)	<20/100	(-)/(-)
	B	>7.5	3.8	<1	(-)/(-)	<20/100	(-)/(-)

(1) Tests performed in duplicate

Facility A also presented high counts of *Enterobacteriaceae* in paprika (EU, 2004). Paprika showed high APC in both facilities. Spices may carry an indigenous microflora that contributes significantly to the microbial load of the sausage batter, though fresh garlic may exhibit some antimicrobial effect (Hew *et al.*, 2006). It has been shown (Kamden *et al.*, 2007) that spices may have an

important role of in the control of *L. monocytogenes* in Italian sausages. Even if the smoking process contributes to reduce the number of indicator and pathogenic microorganisms, as previously observed, the microbiological safety of "Chouriça" and "Salpicão" cannot be assured if highly contaminated raw materials are used (Ferreira *et al.*, 2009).

3.1.3 Non-pathogenic bacteria during the smoking process

These results are summarized in Tables 3.4 and 3.5. Lactic Acid Bacteria developed rapidly in the early stages of fermentation and remained approximately constant throughout the process, in both "Salpicão" and "Chouriça".

Table 3.4: Microbiological characterization of "Salpicão de Vinhais" during smoking process

				t (weeks)					
		Lot	**Seasoning**	**0**	**1**	**2**	**3**	**4**	**5**
MRS counts (Log CFU/g)	**A**	**1**	2.9± 0.2	n.a.	6.5± 0.1	6.8± 0.1	7.0± 0.1	7.39	6.8± 0.5
		2	3.9±0.2	7.9± 1.4	8.7± 0.2	8.6± 0.2	8.5 ±0.4	8.5± 0.3	8.2± 1.0
	B	**1**	7.7± 0.4	7.5± 0.1	7.2± 0.9	7.8± 0.6	8.7± 2.0	8.5±0.0	---
		2	4.2± 0.1	5.0± 0.2	7.8± 1.4	8.0± 0.1	8.6± 0.2	9.0± 0.1	---
M17 counts (Log CFU/g)	**A**	**1**	3.6±2.1	n.a.	6.6± 0.1	5.9± 0.3	7.1± 0.0	7.2± 0.0	5.8± 0.3
		2	6.5± 0.1	7.9± 1.1	7.6± 0.3	7.8± 0.1	6.8± 0.3	7.4± 0.5	7.1 ±0.0
	B	**1**	n.a.	8.4± 0.2	8.5± 0.0	7.6± 0.2	7.9± 1.2	7.6± 0.0	---
		2	4.3± 0.0	6.1± 1.0	7.8± 1.3	7.8	7.9± 0.1	7.9± 0.2	8.9± 0.1
Moulds and Yeasts (Log CFU/g)	**A**	**1**	2.2± 0.1	n.a.	2.0± 0.6	2.2± 0.2	3.5± 0.2	2.3 ±0.2	2.3± 0.2
		2	2.1± 0.0	2.6± 0.9	2.6 ±0.6	2.4± 0.0	3.4± 0.1	2.9± 0.1	3.1± 0.1
	B	**1**	n.a.	5.1± 0.0	2.0± 0.6	2.30±0.1	2.9± 0.3	2.1± 0.0	---
		2	0.8± 1.2	1.6± 0.2	1.8± 0.1	1.20	1.9± 0.1	1.9 ±0.1	1.7 ±0.2

Table 3.5 : Microbiological characterization of "Chouriça de Vinhais" during smoking process

				t (weeks)				
		Lot	**Seasoning**	**0**	**1**	**2**	**3**	**4**
MRS counts (Log CFU/g)	**A**	**1**	3.9± 0.4	n.a.	7.7± 0.0	7.5± 1.3	8.0± 1.0	---
		2	3.7± 0.2	6.4± 0.2	8.9± 0.7	8.6± 0.2	8.4± 0.3	7.9± 0.1
	B	**1**	4.0± 0.0	7.4± 0.4	7.5± 1.3	7.8± 0.0	8.3± 0.4	---
		2	4.4± 0.5	6.9 ±0.2	8.5± 0.7	8.4± 0.9	8.5± 0.3	---
M17 counts (Log CFU/g)	**A**	**1**	4.8± 0.3	n.a.	7.8± 0.0	7.8± 0.2	7.8± 0.2	---
		2	6.2± 0.0	7.3 ±0.2	7.7 ±01	7.9± 0.0	7.9± 0.0	6.9± 0.9
	B	**1**	4.3± 0.5	8.6± 0.0	8.6 ±0.0	7.9 ±0.1	7.3± 1.0	---
		2	4.3± 0.5	7.4± 0.2	8.1± 0.6	7.6± 0.8	7.6± 0.3	---
Moulds and Yeasts (Log CFU/g)	**A**	**1**	2.4± 0.1	2.0± 0.1	1.8± 0.4	2.3± 0.2	2.3± 1.0	---
		2	2.0± 0.1	2.8± 0.0	3.7± 0.1	2.9± 1.1	4.5± 0.2	2.8± 1.1
	B	**1**	1.8 ±0.2	4.9± 0.1	2.6± 0.1	3.9± 0.2	2.9± 0.2	---
		2	2.0± 0.9	2.5± 0.3	4.3± 0.3	3.6± 0.5	4.4± 0.4	---
***E.coli* (Log CFU/g)**	**A**	**1**	0.6± 0.8	2.5± 0.0	<1	<1	<1	<1
		2	2.5± 0.2	<1	<1	<1	<1	<1
	B	**1**	2.4± 0.2	<1	<1	<1	<1	<1
		2	1.4± 0.2	<1	<1	<1	<1	<1

It has been commented that traditionally manufactured sausages have more desirable organoleptic characteristics than those produced on an industrial scale, due to the composition and metabolic activity of the indigenous microflora (Chevallier *et al.*, 2006; Drosinos *et al.*, 2007). Moulds and yeasts were also present during all the stages of the smoking process and they were present in the final product, for both producers A and B. In addition to LAB, yeast and moulds also play an important role in the development of the organoleptic characteristics of fermented sausages (Villani *et al.*, 2005, 2007; Comi *et al.*, 2005; Drosinos *et al.*, 2007), though this can be an indicator of poor hygienic operating conditions.

3.2 Analysis of water activity, pH, D-lactic acid, L-lactic acid concentration:

3.2.1 During the smoking process

The fermentation process that occurs during smoking, due to LAB, produces organic acids, of which lactic acid is predominant (Benito *et al.*, 2007). During the smoking process, there was an increase of both isomers D/L lactic acid, in both "Salpicão" and "Chouriça" (Fig. 3.1), consistent with the previously stated growth of LAB. Genera of LAB such as *Streptococcus*, *Lactococcus*, *Enterococcus* and *Carnobacterium* produce over 90% of the L(+)- isomer as an end product of sugar fermentation. *Leuconostoc* spp. and *Lb. delbrueckii* (all subspecies) on the other hand produce D(-)-lactic acid (Holzapfel *et al.*, 2004). The nature of the lactic isomer is of concern, since high levels of the D(-)-lactic acid isomer are not hydrolysed by lactate dehydrogenase (LDH) in humans and are, thus, capable of causing acidosis (Holzapfel *et al.*, 2004). WHO recommendations indicate a maximum daily intake of 100 mg/kg body weight of this nonphysiological lactic acid isomer (WHO, 1968). There are, however, no recommended limitations for the intake of the L(+)-lactic acid isomer (Holzapfel *et al.*, 2004). The statistical analysis indicates that the values of D-lactic acid are significantly lower than the values of L-lactic acid ($P<0.05$). High D-lactic values may be a health problem for specific patients (Uribarri *et al.*, 1998). The values for the D- lactic acid isomer is not statistically different between the products "Salpicão" and "Chouriça" and the values for the L-lactic acid isomer is not statistically different between the products "Salpicão" and "Chouriça".

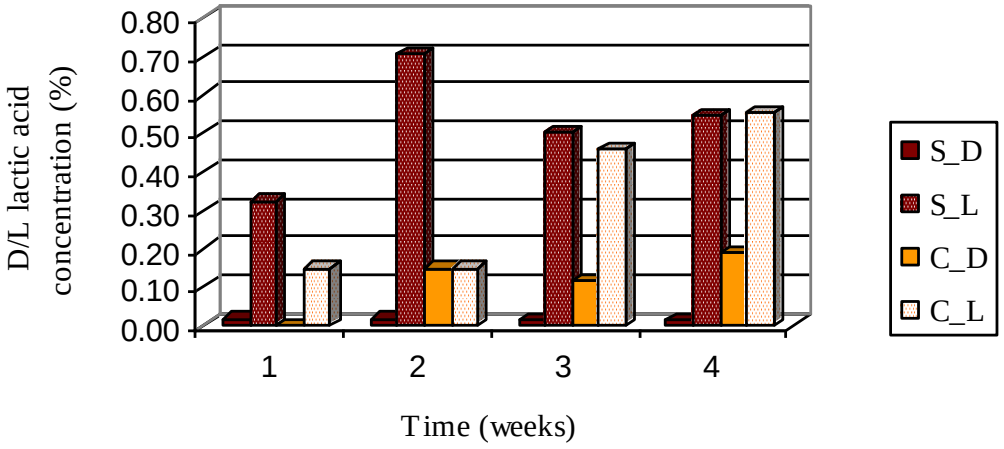


Fig.3.1: Evolution of the concentration of D/L isomers of lactic acid during the smoking step of "Salpicão" and "Chouriça". S_D and S_L being the concentration in "Salpicão" of D-lactic acid and L-lactic acid, respectively; C_D and C_L being the concentration in "Chouriça" of D-lactic acid and L-lactic acid, respectively;

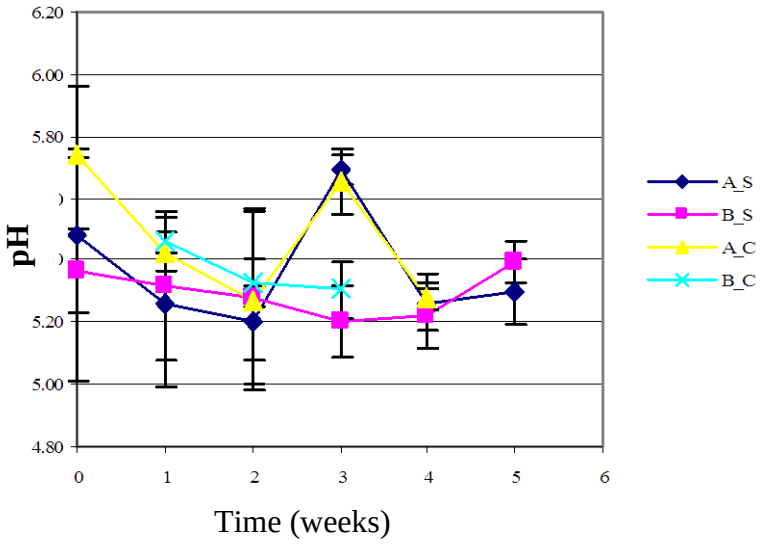
This is not surprising since they are made, essentially, from the same raw materials, differing in the size of pieces of pork used and calibre of sausage produced, which seem not to influence this characteristic of the products.

The production of lactic acid does not translate into a decrease in pH, due to the buffering capacity of the sausage ingredients, namely the meat component (Antwi *et al.*., 2007). These authors suggest that the inhibitory effects of undissociated lactic acid and pH on microorganisms depends on the buffering capacity if bacterial growth is to be inhibited. During the smoking process, along with the growth of LAB and other microflora, the organic acid production, changes in pH, in moisture content and a_w occur, since a drying process also occurs, with the

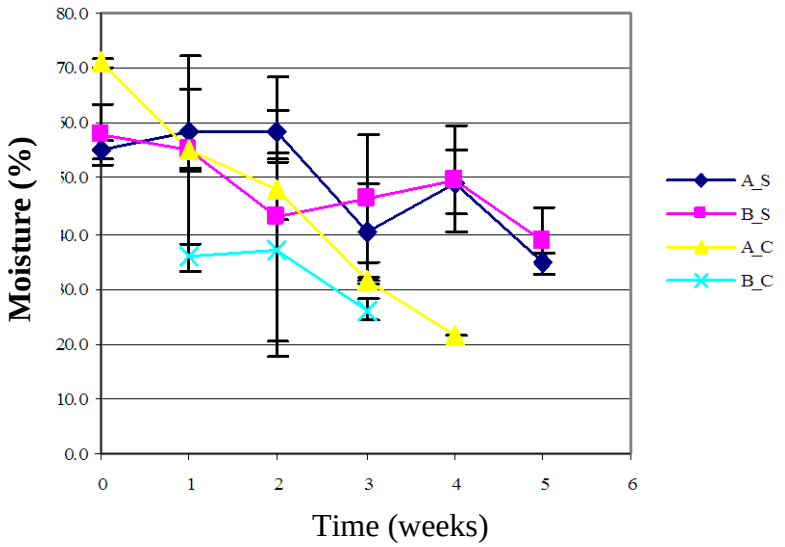
concomitant concentration effect on sodium chloride content as depicted in Fig.3.2.

A slight initial decrease was observed in pH, but the level was never below 5.0. Similar results were reported in Italian sausages (Parente *et al.*, 2001, Urso *et al.*, 2006), in Iberian dry cured sausages (Benito *et al.*, 2007; Casquete *et al.*, 2011), "androlla", from northwestern Spain (Garcia Fontán *et al.*, 2007), and Portuguese "paio alentejano" (Latorre-Moratalla *et al.*, 2010). The values found are higher than those indicated for Spanish "chorizo", which includes a fermentable sugar in its formulation (Salgado *et al.*, 2006).

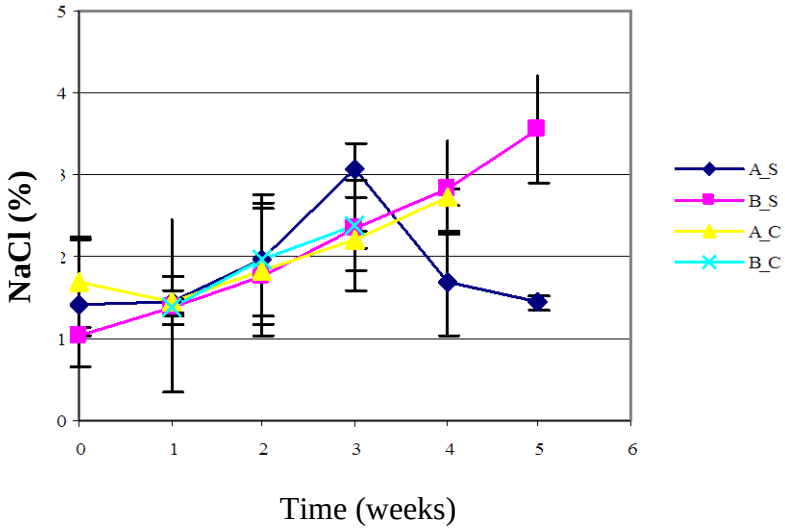
a)



b)


Moisture (%)
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
1
2
3
4
5
6
Time (weeks)
A_S
B_S
A_C
B_C

c)


NaCl (%)
5
4
3
2
1
0
0
1
2
3
4
5
6
Time (weeks)
A_S
B_S
A_C
B_C

d)

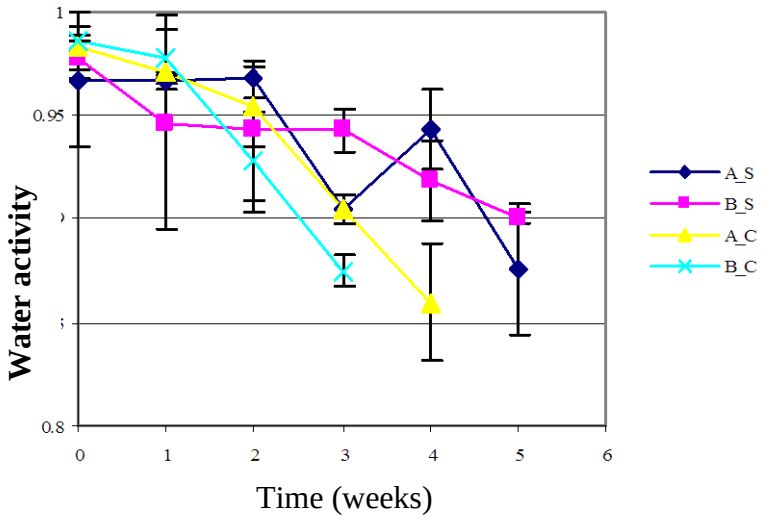


Fig 3.2 Evolution during smoking of a) pH, b) moisture, c) sodium chloride content, water activity: A and B are the two different producers; S and C, "Salpicão" and "Chouriça" respectively. The error bars represent the standard deviation.

Other authors have attributed the slight rise in pH at the end of the process both to proteolytic microbiota (Gonzalez-Fernandez *et al.*, 2003; Fernandez-Lopez *et al.*, 2008) and to the action of endogenous proteases, with the consequent formation of peptides, free amino acids, amines, ammonia, etc. Traditional dry fermented meat products undergo a slow fermentation and pH drop that is not as significant as for other fermentative processes. In Northern countries and North America, fermentation processes can undergo relatively high drying temperatures (above 25 ºC), which enhance the growth of starter cultures and a rapid pH fall (Houben and van't Hooft., 2005, Klingberg *et al.*, 2005). This results in a rapidly stable final product, referred by Houben and van't Hooft (2005) as semi-dry fermented sausages as the pH ranges from 4.0 to 5.0 and shorter ripening periods that result

in a relatively high a_w (> 0.92). In contrast, fermented sausages produced in Southern Europe (Portugal, Spain, Greece) are characterized by the onset of a fermentation by autochtonous bacteria (Ammor *et al.*, 2005; Benito *et al.*, 2007), from the environment or raw materials including spices (Chevallier *et al.*, 2006, Ferreira *et al.*, 2006, Hew *et al*, 2006), higher final pH values (around 5.5), with longer ripening periods, leading to an a_w of less than 0.90 (Barbuti and Parolari, 2002).

The microbial stability of dry sausages is determined by the combination of different factors referred to as the ´hurdle concept` (Tyopponnen *et al.*, 2003; Arnau *et al.*, 2007; Thomas *et al.*, 2008). Raw meat in the batter is kept refrigerated while it absorbs the salt; the salt decreases the initial a_w inhibiting, or at least delaying the growth, of many bacteria; though it is favourable to the growth of halotolerant staphylococci. During the fermentation, which occurs during the smoking process, high levels of LAB produce considerable amounts of lactic acid, lowering, although slightly, the pH value (5.0) of dry sausages. The decrease in pH is not as relevant as it is in more controlled fermentations. One reason for this may be the action of yeasts (brought in by raw meat), which play an important role in proteolysis and lipolysis to develop aroma during the manufacturing process and use lactic acid as substrate at the end of the drying step (Thévenot *et al*, 2005a). At lower pH values, the water holding capacity of meat decreases, increasing the rate of the drying process (Tyopponnen *et al.*, 2003) and lowering the water activity of the product. The germination of *Bacillus* and *Clostridium* spores, which may be derived from spices (and to lesser extent from

meat) are controlled by low pH and a_w (Doyle *et al*, 2001). *Staphylococcus aureus* is tolerant of the dry sausage environmental factors, but it is a fairly poor competitor to LAB. However, the hurdles present in dry fermented smoked sausages are not sufficient to prevent the survival of *L. monocytogenes* (Tyopponnen *et al*., 2003; Arnau *et al*, 2007).

Statistical comparison by two way analysis of variance indicates that no statistically significant differences were found between results from "Salpicão and "Chouriça", neither between producers A and B ($P \geq 0.05$), for the evolution of pH, moisture content, NaCl and a_w during the smoking process.

3.2.2 In the final product

As demonstrated above, the differences between data from "Salpicão" and "Chouriça" are not statiscally significant. So, the following results refer to the joint values of both "Salpicão" and "Chouriça". The data relating with the final product (not distinguishing between "Salpicão" and "Chouriça") will be the input used in the following exposure assessment of the product "Traditional dry fermented, smoked sausage", identified as TDFSS. It is pointless and less valid from a statistical approach to duplicate the calculations, for two products that behave similarly, with fewer samples, which would mean higher errors.

Histograms in Fig.3.3 represent the experimental results from the analysis of 67 samples of traditional dry fermented smoked sausage (except for lactic acid that was analysed in twenty samples).

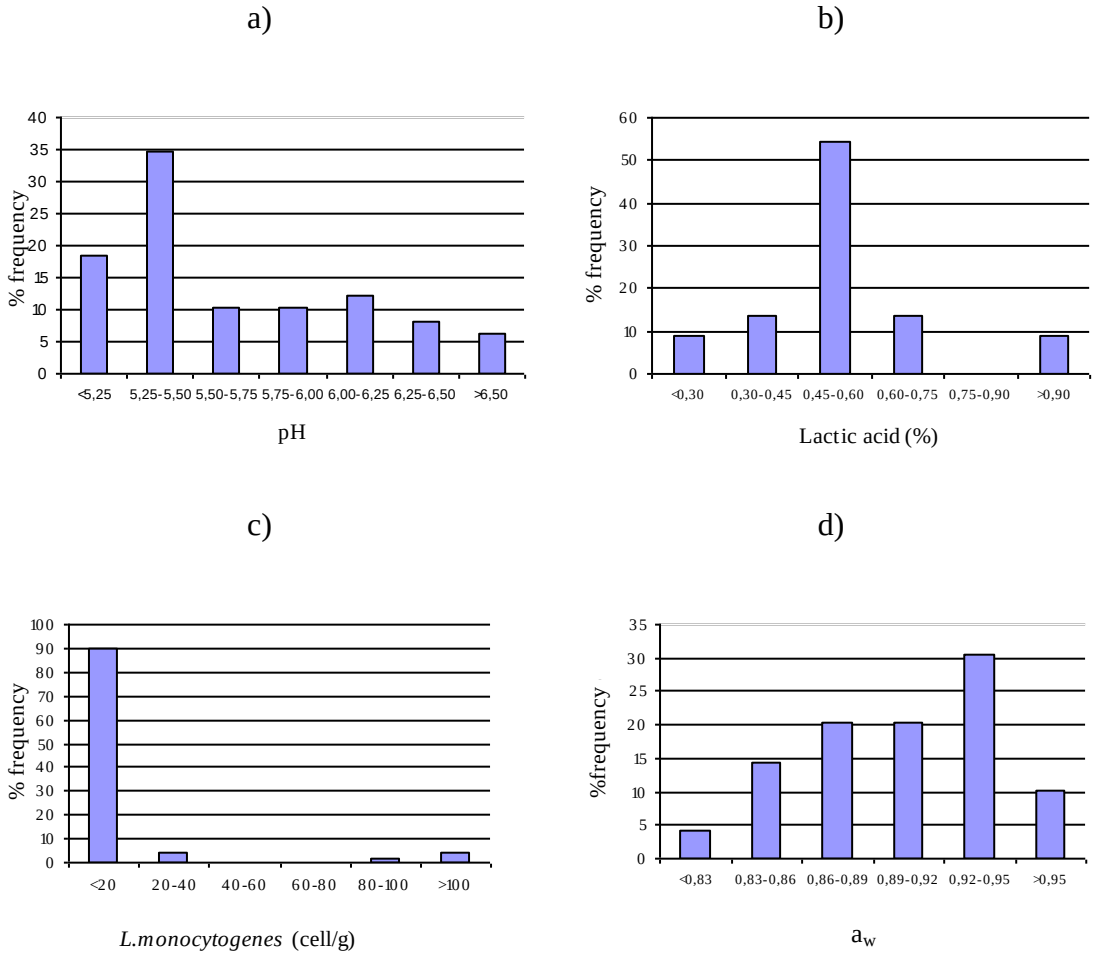


Fig. 3.3 Histograms of (a) pH, (b) lactic acid concentration, (c) *L. monocytogenes* concentration and (d) water activity in the final product "Traditional dry fermented, smoked sausage".

The median, (corresponding to 50% cumulative frequency interval), pH was measured in the interval 5.25-5.50. Another study (Ferreira *et al.*, 2009) reported pH values ranging from 5.3 to 5.4, for these types of sausages. More than 50% of samples presented total lactic acid content in the range 0.45-0.60%. The observed values are similar to those found in pork sausages (0.46 ±0.23), reported by Capita *et al.* (2006), and ''chorizo de cebolla'' (0.36 to 0.90) (Castaño *et al.*, 2002).

Listeria. monocytogenes was not detected by Vidas or direct count techniques, only by the most probable number technique, in 90% of the samples (considering each replicate as a result, as these data will be fitted to a distribution later), though higher concentrations were detected, as previously described in this work. This is a similar result to the study conducted by Ferreira *et al.* (2009), where *L. monocytogenes* was not detected in 96% of the samples.

In Fig. 3.3 (d), it can be observed that over 40% of samples present a_w values higher than 0.92. In chorizo, a semi-dry raw pork meat sausage Mexican style, the samples analysed revealed a wide range of a_w levels (0.81 to 0.97) (Hew *et al*, 2005). Salgado *et al* (2006) determined an a_w of 0.836±0.082 for "Chorizo de cebolla". "Androlla" sausage presented an a_w of 0.902 ±0.041 (Garcia Fontan *et al.*,2007) while Di Cagno *et al* (2008) measured a_w values between 0.87 and 0.89, for Italian PDO sausages. According to Tyopponnen *et al*, (2003), products classified as dry fermented sausages should present a_w values inferior to 0.90. The EC Regulation 2073/2005 (EC, 2005) classifies as unable to support growth of *L.monocytogenes*, food products with a_w equal or inferior to 0.92.

3.3 Risk assessment of *L. monocytogenes* in product "Traditional dry fermented, smoked sausage".

The objective of this study was not to produce an accurate risk assessment, because several assumptions and hypothetical values were used. The main objective was to work with the available data, previously obtained in sections 3.1

and 3.2, and assess the probability of illness caused by consumption of Traditional dry fermented, smoked sausage contaminated with *L. monocytogenes,* in Portugal and how the incidence of listeriosis might be reduced (section 3.4). Due to the rare development of microbiological risk assessment in Portugal, this study was undertaken with the aim to develop a quantitative risk assessment model to provide a more accurate prediction of cases of listeriosis by consumption of contaminated traditional dry fermented smoked sausages. In this case, focusing the microbiological problem on a small Portuguese region, has been useful for obtaining more realistic data and for identifying the gaps or limited information. A probabilistic approach was used in part of the model with respect to Exposure Assessment, whereas in the Hazard Characterization a deterministic approach was used. The final result is, therefore, a probabilistic estimation of risk.

3.3.1 Hazard identification

After a decline in the 1990s, coinciding with a reduction in the prevalence and concentration of *L. monocytogenes* in RTE foods, the number of cases of human listeriosis has been increasing since 2000 in Europe, with several cases being reported (EU, 2007). The disease is associated with pregnancy, but is now predominantly associated with immuno-compromised persons amongst the older section of the population (over 60 years). Extensive hazard identification can be consulted in "Scientific Opinion of the BioHazard Panel on *L .monocytogenes* risk related to ready-to-eat foods" (EU, 2007), in the "Quantitative Assessment of Relative Risk to Public Health from Foodborne *L. monocytogenes* Among

Selected Categories of Ready-to-Eat Foods", (FDA/FSIS, 2003) and in the "Risk assessment of *L. monocytogenes* in ready-to-eat foods, (FAO/WHO, 2004).

Traditional dry fermented meat sausages are considered to be shelf stable products, with low a_w (Lücke, 1998; FDA/CFSAN, 2003; FAO/WHO, 2004). Nevertheless, the presence of *L. monocytogenes* is recurrent, being detected in 22.4% of the samples analysed (section 3.1-during process and in final product). In section 3.1, the prevalence and concentration of the pathogen of concern in traditional dry fermented meat sausages is discussed. The risk assessment considered only food safety risks emanating from systemic listeriosis, the manifestations of which are serious and, in 20–30% of cases, fatal. Full details of the hazard presented to consumers by *L. monocytogenes* are presented in FAO/WHO (2004).

3.3.2 Exposure assessment

There are very few data which describe the level of contamination of foods with *L. monocytogenes* at the point of consumption. To overcome this limitation, the conceptual model for Exposure Assessment described in Material and Methods, section 2.3.1 was used. Luber *et al.* (2011) commented that growth/no-growth characteristics of food are increasingly becoming the focus of *L. monocytogenes* microbiological criteria, especially where the regulator has decided to adopt the two level approach recommended by *Codex Alimentarius*. These models are valuable tools in assessing the risk of growth of *L. monocytogenes* in food products as affected by formulation, process and storage conditions (EFSA,

2007). On January 1st 2006, the Commission Regulation 2073/2005 (EC, 2005) became effective for all the European Union States. In this legislation, the microbiological criteria regarding *L. monocytogenes* in RTE foods are differentiated according to three major characteristics:

i) target population - i.e. infants or people with special medical conditions, for whom foods are required to be free of *L. monocytogenes* (absence in 25g in a 10 unit sampling plan);

ii) RTE foods, other than those intended for (i) and that are unable to support growth of *L. monocytogenes*;

iii) RTE foods, other than those intended for (i) and that are able to support growth of *L.monocytogenes*;

The EC Regulation 2073/2005 (EC, 2005) classifies as RTE foods that are unable to support growth:

a) products with pH ≤ 4.4 **or** a_w ≤ 0.92;

b) products with pH ≤ 5.0 **and** a_w ≤ 0.94;

c) products with a shelf-life of less than five days.

When plotting the a_w and pH values of the samples obtained in 3.2.2, for the final product of traditional dry fermented smoked sausage and applying the prior classification, the following graphic is obtained (Fig. 3.4).

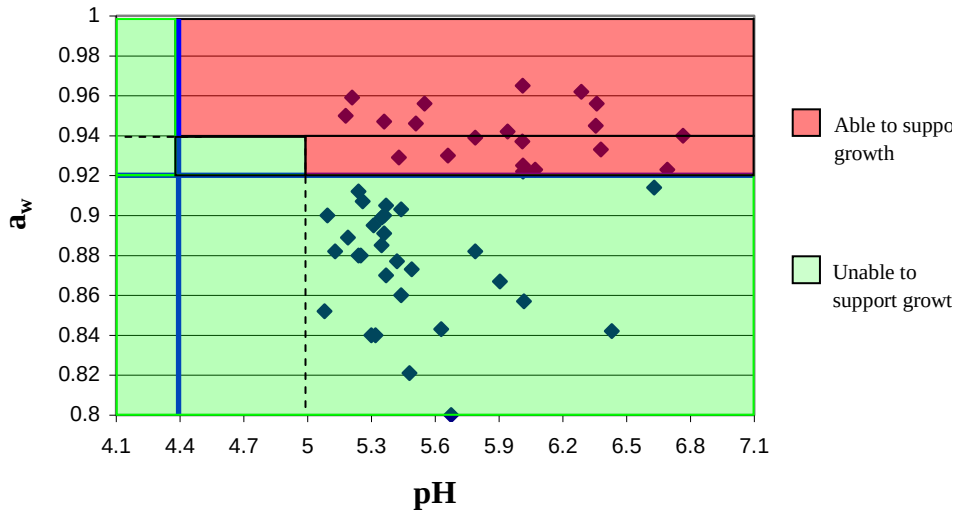


Fig.3.4 Plotting of pH and a_w results of the final product for traditional dry fermented smoked sausage, and comparison with EC Reg.2073/2005 (EC, 2005) criteria.

Figure 3.4 indicates that based on the binomium pH and a_w, 39% of the contaminated samples **are able** to support growth of *L. monocytogenes*. This contradicts the assumption that dry smoked fermented meat products are unable to support the growth of the pathogen and may even reduce its numbers during storage (FDA/FSAN, 2003; Drosinos *et al*, 2005; Lindqvist and Lindblad, 2009). In the Scientific Opinion of the Panel on Biological Hazards, the European Food Safety Agency (EU, 2007) compares four growth/no growth curves from literature with the EC Regulation 2073 criteria, at 4 ºC and 10 ºC, concluding that not only pH and a_w, but also storage temperature defines the ability of a food to support growth. However, Regulation 2073/2005 (EC, 2005) does not include a clear guideline regarding the temperature at which the industry should evaluate the

ability of its products to support the growth of *L. monocytogenes* (Koutsoumanis and Angelidis, 2007). In the FAO/WHO (2003) risk assessment it seems that the potential for growth strongly influences risk, in RTE products, though the extent of that increase is dependent on the characteristics of the food and the conditions and duration of refrigerated storage. The ability of these RTE products to support growth of *L. monocytogene*s appears to increase the risk of listeriosis per serving by 100- to 1000-fold over what the risk would have been if the foods did not support growth. This supports the importance of integrating growth/no growth models in the final exposure assessment model. These results indicate that traditional dry fermented smoked sausage may not be shelf-stable for all products that are being consumed, at the moment, in Portugal, and it depends on the temperature conditions they are exposed to. The cause of this is the artisanal manufacturing characteristic of the product, with variable calibre, empirical control of drying, during the smoking process (based on the experience of the operator), non-controlled smoking conditions, depending on climatic conditions such as temperature and relative humidity. It is possible to say that the characteristics of final products of traditional dry fermented smoked sausage, fall near the boundaries that allow, or not, growth of *L. monocytogene*s; thus, a description of its behaviour is necessary. In this section, a probabilistic approach was used.

i) Growth/No growth interface model and binomial transformation.

A model of the interface between growth or no growth conditions was developed, previous to the calculation of growth, based on the values of a_w, pH and temperature, according to described methodology.

The coefficient estimates (*ai*) of parameters and statistical indices of the logistic regression model are presented in Table 3.6 and its statistics in Table 3.7.

As determined by the concordance index, the degree of agreement between the predicted probabilities and the observations was 98.3%, indicating a good fit to the observed data.

Table 3.6: Parameter estimates of the logistic regression model for growth/no growth interface with *L.monocytogenes*

Parameter	Estimated value	Standard Error
Intercept	-519.46	246.67
T	-6.12	1.87
pH	-54.76	23.71
a_w	1423.77	460.75
T^2	-0.0177	0.0053
pH^2	0.9213	0.9389 (n.s)
a_w^2	-866.073	227.148
$T \cdot pH$	1.31	0.20
$T \cdot a_w$	-0.15	1.27 (n.s)
$pH \cdot a_w$	41.17	16.44

n.s.: Not significant ($P > 0.05$)

Table 3.7: Statistical parameter of the logistic regression model for growth/no growth interface with *L. monocytogenes*

Statistical parameter	
Hosmer-Lemeshow	11.85 (df = 8, P = 0.158)
Concordant rate (%)	98.3
Discordant rate (%)	1.6
False positive	30/900 (3.3%)
False negative	31/900 (3.4%)

The Hosmer-Lemeshow test is a commonly used test, similar to chi-square goodness of fit test, for assessing the goodness of fit of a model. If the Hosmer–Lemeshow statistic takes a low value, close to zero, or if its corresponding P-value is high, the model fits the data well (Gysemans *et al.*, 2007). The goodness-of-fit of the model based on the data set is reasonable, as is indicated by the Hosmer–Lemeshow statistic. The goodness of fit could be improved by the removal of a possible outlier (Koutsoumanis and Sofos, 2005).

The resulting equation, therefore, is:

$$\text{Logit}(Pg) = -519.46 - 6.12T - 54.76pH + 1423.77a_w - 0.0177T^2 - 866.073a_w^2 + 1.31T \cdot pH + 41.17pH \cdot a_w \quad \text{Eq.(1)}$$

As described in section 2.3.1 of Materials and Methods, the obtained equation is combined with published equations for the calculation of exponential growth rate (μ) and lag phase (Buchanan and Phillips, 2000). Though official organisms like EFSA (2007) state the relevance of growth/no growth boundaries, few risk assessments incorporate this in their exposure assessment models (Koutsoumanis and Angelidis *et al*, 2007). Because the calculation of final concentration of *L. monocytogenes*, using the previously mentioned equations, was achieved using a

probabilistic approach, the several input variables had to be described, where possible and justifiable, by a probability distribution, as described below.

ii) Input distribution functions to be used in the Exposure Assessment model.

Table 3.8 summarizes the inputs used in the Exposure Assessment model. Although some studies (Bemrah *et al*., 1998; FDA/FSIS, 2001) indicate that *L. monocytogenes* is probably heterogeneously distributed in some foods, this exposure assessment assumed the simplification that pathogens are distributed homogeneously within a food. Furthermore, all *L. monocytogenes* food isolates were accepted as having the same potential to cause human illness (FDA/FSAN, 2003). The distribution of the counts of organisms in log CFU/g that best fitted both quantitative and qualitative data is a log-normal distribution with -5 as mean and 1.5 as standard deviation. This indicates lower contamination values when comparing to frequency distribution of observed contamination levels in Parma ham which was fitted by a log-normal distribution with the mean -4.8 and standard deviation 1.9 (Giovannini *et al.*, 2007).

The available data on *L. monocytogenes* levels had some limitations that affected the distribution for levels of *L. monocytogenes* in foods, as there are relatively few data points above the limit of detection (0.04 cfu/g). Few data may result in biased estimations (FDA/FSAN, 2003). The contamination distribution was obtained as described in Material and Methods, section 2.3.1.1.

Another simplification was assumed by truncating the distribution at an upper level of 1000 CFU/g as this was the maximum value of contamination observed,

avoiding overestimating calculations in the upper tail (Nauta *et al.,* 2009; Garrido *et al.,* 2010).

Table 3.8 Distribution functions of product characteristics (pH and a_w), kinetic parameters (N_0 and T) and point estimates used in model and Latin hypercube simulation of populations of *L. monocytogenes*

Step or parameter	Variable	Distribution and description	Mean	90% interval
Final product, after smoking (potentially until consumption)	pH	Inverse-Gauss (0.74, 1.51); shif=4.92	5.66337	5.142-6.667
	a_w	Triangular (0.78408, 0.96500, 0.96500)	0.90469	0.8245-0.9604
Initial population (logCFU/g)	N_0	Normal (-5, 1.5) truncate (3)	-5,0001	-7.47-(-2.53)
Temperature during storage and transportation (ºC)	T	Weibull (6.62,18.4); shift=-3.96	15.196	9.66-20.87
Time during shelf life	CP1	Normal (20,7); truncate (0 to 90)	20	8.49-31.51
Time at the end of shelf life	CP2	90 days	--	--
Lag time	lag	(Buchanan and Phillips, 2000)	0.0557	2.450E-09-0.009998
Exponential growth rate	μ	(Buchanan and Phillips, 2000)	0.00565	3.370E-05-0.02337
Population of *L. monocytogenes* in the batter	N_b	$Nb=N_0+(30/t_{4D1})x4$	-3.769244	(-6.237500) - (-1.30198)
Population of *L. monocytogenes* in the final product with the use of the bac + strain **HA6111-2**	$N_0^{'}$	$N_0^{'}=N_b-(30/t_{4D2})x4$	-7,7346	-10.24-(-5.31)

iii) Consumption data

Because the risk characterization intends to distinguish risk by subpopulation, the exposure assessment output should be discriminated to depict variation in exposure among various sub-populations (FAO/WHO, 2009). The consumption patterns of sausages in the region of Porto are summarized in Table 3.12. A worst case situation was considered, so the upper limit of the bin referring to the category of consumption was the one considered (for instance, for the interval bin "one to three times per week" it was assumed the value of three servings per week).

Table 3.9 Consumption patterns of sausages in the region of Porto (Lopes *et al*., 2006)

	Total population		**Elderly population**	
	Absolute frequency	Relative frequency	Absolute frequency	Relative frequency
Never or less than once per month	414	17.3%	199	34.0%
One to three times per month	385	16.1%	108	18.5%
Once per week	582	24.3%	125	21.4%
Two to Four times per week	683	28.5%	113	19.3%
Five to six times per week	118	4.9%	18	3.1%
Once per day	199	8.3%	22	3.8%
Two to three times per day	17	0.7%	0	0.0%
Four to five times per day	0	0.0%	0	0%
Six or more times per day	0	0.0%	0	0.0%
	Total	100.0%	Total	100.0%
Sausages consumption (g/day)				
Average:	6.1		3.7	
Stdev:	6.9		5.0	
	Total: 2398 between 18 and 92 years old, both sexes		Total: 585 between 65 and 91years old, both sexes.	

http://higiene.med.up.pt/consumoalimentarporto/home.php?var=mensagem (accessed 25 November 2008)

Assuming 30 days per month, 52 weeks per year, 365 days per year it was possible to calculate the number of servings, *per person*, *per year*.

3.3.3 Hazard Characterization

Hazard characterization integrates information and data acquired during the hazard identification and exposure assessment into a qualitative and/or quantitative evaluation of the nature of the adverse effects associated with the consumption of traditional dry fermented smoked sausage.

The Exposure Assessment used the information of initial contamination of final product N_0, a_w, pH, temperature exposure, in the already described models, and generated an output of frequency distribution of simulated prevalence of *L. monocytogenes* in traditional dry fermented smoked sausage, at a given point of Shelf-life (CP1), and at the final point of Shelf-life (CP2). Knowing the serving size, the simulated dose at consumption of one serving can be calculated; the Hazard Characterization, knowing the dose and the adequate r-value, calculates the probability of each serving causing listeriosis; finally, using information regarding consumption data, by each sub-population, the number of listeriosis cases per year can be estimated. Three population subgroups were considered, based on the ‘‘Risk assessment of *L. monocytogene*s in RTE foods, Technical Report” (FAO/WHO, 2004). Other authors have adopted to differentiate between susceptible subgroups (Giovannini *et al.*, 2007, Garrido *et al.*,, 2010). The Intermediate-age group includes susceptible populations like imunocompromised patients, for whom there are insufficient data to consider as a separate sub-

population. The Perinatal population is a susceptible population that includes dead fetus and neonates, giving the total number of pregnancies, successful or not, using the data from the "Estatísticas Demográficas 2007" Portuguese report (INE, 2008). Values for r, for the three populational sub-groups considered were obtained from ''Risk assessment of *L. monocytogenes* in ready-to-eat foods, Technical Report" (FAO/WHO, 2004). The effect of approximating the consumption data of Porto to the consumption data of the Portuguese population on the accuracy of the results will be discussed in 3.3.4 (see Table 3.11). The results are summarized in Table 3.10.

Table 3.10 Consumption data and "r-value" for three population subgroups (FAO/WHO, 2004)

	Intermediate age	**Elderly (>65)**	**Perinatal**
% total population	79%	20%	1%
Mean serving size (g)	6	4	6
Number of servings/person/year	113	71	113
r- value	5.34E-14	8.39E-12	4.51E-11

Such division in population sub-groups is largely subjective and arbitrary (McLauchlin *et al.*, 2004). It attempts to incorporate part of the host component of the dose–response relationship into the model. The exponential dose–response curve was chosen (FAO/WHO, 2003; Giovannini *et al.*, 2007; Ross *et al.*, 2009a, b, c; Garrido *et al.*, 2010). This model assumes that (i) the probability of infection has a constant value for any given host and any given strain of the pathogen, and (ii) the distribution of the organisms in the inoculum is random, and characterized

by a Poisson distribution (FAO/WHO, 2003). The first assumption does not take account of the biological variability of both hosts and pathogen strains. The second assumption excludes spatial clustering of cells in the inoculum. Clustering, however, has little effect on the shape of the dose–response relationship (Giovannini *et al.*, 2007). Strain variation has been incorporated in published work (Devlieghere *et al.*, 2001; Ross *et al.*, 2009c). This model assumes each ingested cell acts independently, and all cells have the same probability of causing infection. The non-threshold assumption implies the existence of some level of risk for any dose greater than zero (FAO/WHO, 2009). Effectively, the FDA/CFSAN (2003) exposure assessment represents the most complete estimate of the exposure of a population to food-borne *L. monocytogenes* (Ross *et al.*, 2009 c). This model was adopted, and further refined, by FAO/ WHO (2004). Due to its broader data-base, it is probably the most-preferred of the *L. monocytogenes* dose–response models currently available (Ross *et al*, 2009 c). Since it was not possible to obtain an r-value from outbreaks in Portugal, the same r-values used by FAO/WHO (2004) were used in the present risk assessment. A significant weakness of the dose–response model used is that it relies on the validity of all assumptions and data used. Any change in these, particularly for higher risk products, could significantly affect the estimation process. Due to the difficulties in obtaining new dose–response data, it is unlikely that this component of the model will be improved by research in the near future (Ross *et al*, 2009b).

3.3.4 Risk Characterization

Risk characterization is the integration of hazard identification, hazard characterization, and exposure assessment into an estimate of the adverse effects likely to occur in a given population, this is, the number of cases of listeriosis, per year, in a given population, in this case, the Portuguese population. The estimates for the risk of listeriosis and intermediary calculation are summarized in Tables 3.11 A, B and C.

Table 3.11 Estimation of the total listeriosis cases at two distinct consumption periods (CPs), based on the simulated concentration and prevalence of *L. monocytogenes* (LM) in fermented sausages for intermediate age groups (A), elderly (over 65) and perinatal (C).

A- Intermediate age

Simulated concentration *L.monocytogenes* (logCFU/g)	**Simulated *L.monocytogenes* prevalence (%)**		**Simulated dose at consumption (CFU/serving)**	**Probability listeriosis per serving**	**Predicted nº of listeriosis cases per year**	
	CP1	**CP2**			**CP1**	**CP2**
0.5	96.69	98.56	19.0	1.0132E-12	4.9375E-04	5.0329E-04
1	0.03	0.12	60.0	3.2040E-12	4.8444E-07	1.9378E-06
1.5	0.02	0.13	1.90E+02	1.0132E-11	1.0213E-06	6.6384E-06
2	0.03	0.12	6.00E+02	3.2040E-11	4.8445E-06	1.9378E-05
2.5	0.02	0.07	1.90E+03	1.0132E-10	1.0213E-05	3.5745E-05
3	0.04	0.09	6.00E+03	3.2040E-10	6.4593E-05	1.4533E-04
3.5	0.03	0.11	1.90E+04	1.0132E-09	1.5319E-04	5.6171E-04
4	0.05	0.07	6.00E+04	3.2040E-09	8.0741E-04	1.1304E-03
4.5	0.09	0.08	1.90E+05	1.0132E-08	4.5958E-03	4.0852E-03
5	0.05	0.1	6.00E+05	3.2040E-08	8.0741E-03	1.6148E-02
5.5	0.09	0.02	1.90E+06	1.0132E-07	4.5958E-02	1.0213E-02
6	0.03	0.06	6.00E+06	3.2040E-07	4.8444E-02	9.6889E-02
				Total	**0.1086**	**0.1297**

Considering the established FSO, in Regulation 2073/2005 (EC, 2005), of 2 log CFU/g at the end of shelf life (CP2), 0.60% of the servings exceed it and are responsible for 99.6% of the predicted cases of listeriosis. 0.40% of all servings

are predicted to exceed the FSO at some point of its shelf-life (CP1), causing 99.5% of predicted listeriosis cases *per annum*, for all sub-groups.

B - Elderly

Simulated concentration *L.monocytogenes* (logCFU/g)	**Simulated *L.monocytogenes* prevalence (%)**		**Simulated dose at consumption (CFU/serving)**	**Probability listeriosis per serving**	**Predicted nº of listeriosis cases per year**	
	CP1	**CP2**			**CP1**	**CP2**
0.5	96.69	98.56	12.6	1.9967E-09	7.5934E-04	7.7403E-04
1	0.03	0.12	40.0	6.3140E-09	7.4503E-07	2.9801E-06
1.5	0.02	0.13	1.26E+02	1.9967E-08	1.5707E-06	1.0209E-05
2	0.03	0.12	4.00E+02	6.3140E-08	7.4503E-06	2.9801E-05
2.5	0.02	0.07	1.26E+03	1.9967E-07	1.5707E-05	5.4973E-05
3	0.04	0.09	4.00E+03	6.3140E-07	9.9338E-05	2.2351E-04
3.5	0.03	0.11	1.26E+04	1.9967E-06	2.3560E-04	8.6387E-04
4	0.05	0.07	4.00E+04	6.3140E-06	1.2417E-03	1.7384E-03
4.5	0.09	0.08	1.26E+05	1.9966E-05	7.0680E-03	6.2827E-03
5	0.05	0.1	4.00E+05	6.3138E-05	1.2417E-02	2.4834E-02
5.5	0.09	0.02	1.26E+06	1.9965E-04	7.0680E-02	1.5707E-02
6	0.03	0.06	4.00E+06	6.3120E-04	7.4503E-02	1.4901E-01
				Total	**0.1670**	**0.1995**

C -Perinatal

Simulated concentration *L.monocytogenes* (logCFU/g))	**Simulated *L.monocytogenes* prevalence (%)**		**Simulated dose at consumption (CFU/serving)**	**Probability listeriosis per serving**	**Predicted nº of listeriosis cases per year**	
	CP1	**CP2**			**CP1**	**CP2**
0.5	96.69	98.56	1.90E+01	8.5571E-10	5.2125E-03	5.3134E-03
1	0.03	0.12	6.00E+01	2.7060E-09	5.1143E-06	2.0457E-05
1.5	0.02	0.13	1.90E+02	8.5571E-09	1.0782E-05	7.0083E-05
2	0.03	0.12	6.00E+02	2.7060E-08	5.1143E-05	2.0457E-04
2.5	0.02	0.07	1.90E+03	8.5571E-08	1.0782E-04	3.7737E-04
3	0.04	0.09	6.00E+03	2.7060E-07	6.8191E-04	1.5343E-03
3.5	0.03	0.11	1.90E+04	8.5571E-07	1.6173E-03	5.9301E-03
4	0.05	0.07	6.00E+04	2.7060E-06	8.5239E-03	1.1933E-02
4.5	0.09	0.08	1.90E+05	8.5571E-06	4.8519E-02	4.3128E-02
5	0.05	0.1	6.00E+05	2.7060E-05	8.5238E-02	1.7048E-01
5.5	0.09	0.02	1.90E+06	8.5568E-05	4.8517E-01	1.0782E-01
6	0.03	0.06	6.00E+06	2.7056E-04	5.1136E-01	1.0227E+00
				Total	**1.1465**	**1.3695**

According to the "*Listeria monocytogenes*-Risk Assessment, from the FDA, 2003", food categories were categorized as very high risk (>100 cases per annum), high risk (>10 to 100 cases per annum), moderate risk (>1 to 10 cases per annum) and low risk (≤1 cases per annum). This means, for the Portuguese population, that Traditional Dry Fermented Smoked Sausages constitute:

i) a low risk for the Intermediate Age and Elderly population (less than 1case per annum);

ii) an intermediate risk for the Perinatal populations (1-10 cases per annum);

iii) These results confirm the general premise that Dry Fermented Sausages are low risk products (FDA/FSAN 2003; FAO/WHO, 2004; Drosinos *et al.*, 2005; Ross *et al.*, 2009 c; Garrido *et al.*, 2010).

The higher risk was obtained for the Perinatal population, but 55.6% of all listeriosis cases reported, within Europe, were recorded in patients above 65 years (EFSA, 2007). Ross *et al.*, (2009c) refers to the association of the listeriosis outbreaks with hospital and aged care settings. There is evidence (Torvaldsen *et al.*,1999; Ogunmodede *et al.*, 2005) that pregnant women may reduce their consumption of some foods to reduce their exposure to *L. monocytogenes*.

In order to assess the accuracy of the proposed model, the number of estimated cases would have to be compared with that reported in Portugal, but data relating food borne illnesses in Portugal, is scarce, as there is no oficial epidemiological surveillance program, in place. Listeriosis is not a disease of mandatory notification to authorities in Portugal.

The assumptions behind the present model may clearly affect the validity of the results obtained. Sources of uncertainty, that could affect the risk estimation, must be identified in the construction of the model, including descriptive errors which represent incorrect or insufficient information regarding the assumptions made in the model (Garrido *et al.*, 2010).

Several uncertainty-originating assumptions can be identified in this model (Table 3.12).

The samples of traditional dry fermented smoked sausage were taken from several sources, as previously described. There was no differentiation between samples from different origins. This is an uncertainty factor. Also, for the samples acquired from the market, with some of its shelf-life elapsed, it was assumed that the results referred to the beginning of the shelf life, t=0.

It has been assumed that the whole population consumes sausages throughout the year, with constant frequency and same serving size, but an unknown percentage of the population could have different habits of consumption (for instance, for ethical or health reasons, (Garrido *et al.*, 2010)). Also, the consumption for the region of Porto, though it represents 1.28 million inhabitants (INE, 2008) may not represent the habits of the entire Portuguese population. The study of consumption patterns studied the food product "sausage"; this term is broader and it includes other products besides traditional dry fermented smoked sausages, like frankfurters, for instance. This lack of knowledge is a source of uncertainty for these parameters.

Table 3.12 Main assumptions of the model

Assumptions of the model
No difference in prevalence existed related to the product origin (industrial units, from local market and commercial stores)
All samples were considered to be taken at time zero, i.e. the beginning of shelf life.
The consumer's behaviour (serving size and frequency) is the same throughout the year
The consumption pattern for sausages is the same for traditional dry fermented smoked sausage
The consumption pattern of the population of Porto is the same for the population of Portugal.
Each *L. monocytogenes* strain has the same probability to cause infection (same virulence) and reach the same maximum population.
There was no distinction between the temperature condition previous to purchase (distribution and storage) and posterior to purchase (home storage)
r values for the dose response model, for the Portuguese population are the same used in (FAO/WHO, 2003).

It was assumed that all of the strains isolated from food had the same potential to cause listeriosis, i.e. that all *L. monocytogenes* strains have a similar virulence. The same assumption has been made by several authors, in listeriosis risk

assessment (Aarnisalo *et al.*, 2008; Lindqvist *et al*, 2002; Mataragas *et al.*, 2008; Garrido *et al.*, 2010). But it has been recently stated that there is a need to incorporate intra-species biodiversity in exposure assessment (Augustin, 2011). Buncic *et al.* (2001) reported that in human and food isolates of *L.monocytogenes*, the serovar 1/2a, as a group, tended to be more resistant to antilisterial bacteriocins at 4 °C than the group of isolates of serovar 4b. While there are many sources of uncertainty, Ross *et al.* (2009 b, c) refers to the potential growth of *L. monocytogenes* in processed meats and the probability of infection from ingestion of a given dose of *L. monocytogenes,* as those most contributing to uncertainty in the risk estimates. Besse *et al.* (2006) suggested that due to *quorum sensing*, the effect of the inoculum size of *L.monocytogenes* affects the contamination level attained. Consequently, when performing quantitative risk assessment studies, it is relevant to consider the food contamination levels, which are generally very low, and to integrate the inoculum size effect. Continuous effort should be made to obtain more data for future improvement of the model.

3.3.5 Evaluating Uncertainty and Variability

Due to computational limitations, the evaluation of parameters uncertainty and variability was performed on a subset of 100 × 1000 second-order simulations. Firstly, the uncertainty and variability of the inputs were determined and then propagated in the model (Mataragas *et al*, 2010). This originated one hundred density function curves of the Predicted number of cases of listeriosis, per year. In Figure 3.5, each green line represents one density function curve from one

simulation and the thick black line represents the density function curve of predicted number of cases of listeriosis per year when no parameter uncertainty is considered.

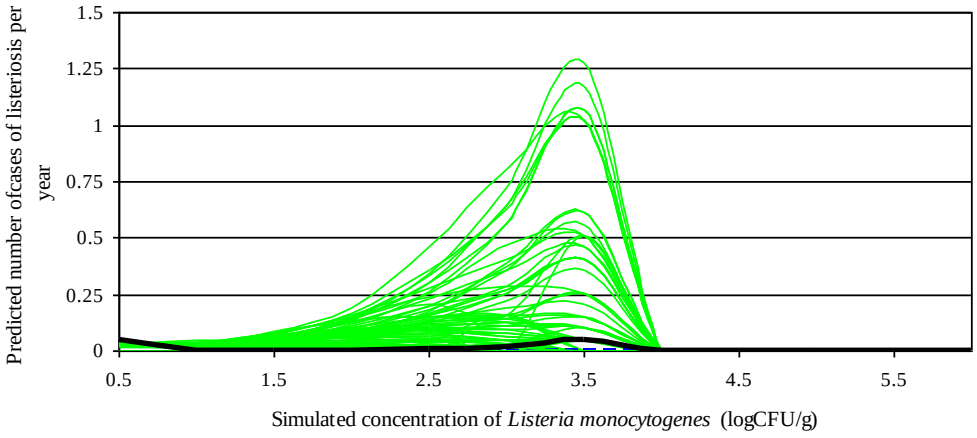


Fig. 3.5 Outcome of the second order analysis showing the Predicted number of cases of listeriosis per year when parameter uncertainty and variability are considered (green line) and when only parameter variability is considered (thick black line).

Second order modelling demonstrated that the contribution of parameter uncertainty is significant as described by the considerably different "spaghetti-like" curves describing the final model output - density function Predicted number of cases of listeriosis per year (Pouillot *et al.*, 2003; Wu *et al.*, 2004; FAO/WHO, 2009). The model output, when no parameter uncertainty was considered, clearly under predicted the number of listeriosis cases per year. Three major conclusions may be taken: first, two dimensional risk assessment is a more informative and complete analysis, thus giving a range of predicted results to the risk management process (Dawber *et al.*, 2009). Nauta (2000) commented that a major outbreak

may be overlooked if the distinction between uncertainty and variability is neglected; second, in this particular study, it is necessary to collect further information on the inputs (T, a_w, pH and N_0), in order to reduce its uncertainty and, therefore, permit a more rigorous risk assessment; third, the model robustness needs improvement.

3.3.6 Sensitivity analysis of the exposure assessment

Sensitivity analysis helps to identify key variables that are potentially controllable and can be used to identify key sources of uncertainty for which additional research or data collection could improve the state of knowledge and thereby reduce ambiguity regarding the characterization of risk and comparison of risk management options (FAO/WHO, 2009).

Table 3.13 Sensitivity analysis of some components of the exposure assessment a) growth rate, b) lag time, c) N predicted at CP1, d) N predicted at CP2

a)

Sensitivity			
Rank	**Name**	**Regr**	**Corr**
#1	a_w	0.608	0.924
#2	pH	0.293	0.260
#3	T	0.249	0.230
#4	Binomial (1,Pg)	0.044	0.203
#5	No	0.000	-0.002
#6	t during shelflife	0.000	-0.015

b)

Sensitivity			
Rank	**Name**	**Regr**	**Corr**
#1	a_w	-0.102	-0.543
#2	pH	-0.032	-0.736
#3	T	-0.027	-0.278
#4	No	0.000	-0.009
#5	t during shelflife	0.000	0.008

#6	Binomial(1,Pg)	0.000	-0.130

c)

Sensitivity CP1			
Rank	**Name**	**Regr**	**Corr**
#1	No	0.784	0.954
#2	Binomial (1,Pg)	0.519	0.270
#3	t during shelflife	0.028	-0.002
#4	pH	0.019	0.019
#5	T	0.017	0.016
#6	a_w	0.012	0.048

d)

Sensitivity CP2			
Rank	**Name**	**Regr**	**Corr**
#1	Binomial (1,Pg)	0.774	0.307
#2	No	0.609	0.941
#3	T	0.003	0.015
#4	pH	0.000	0.017
#5	a_w	0.000	0.054
#6	t during shelflife	0.000	-0.007

The growth rate and the lag time, in the event of the conditions being favourable to *L. monocytogenes* growth, were mainly influenced, as expected, by a_w and then by pH. Regarding the prediction of growth at a given time during shelf-life (CP1), the growth is influenced by the initial contamination N_0 and then, by the possibility of growth determined by the logistic function. The predicted growth at the end of shelf life is mainly influenced by the possibility of growth/no growth, and then by the initial contamination N_0. The inputs that affect the model output more, need to be improved and more data acquired.

3.4 Risk Management Scenarios: Food Safety Objectives (FSO), Process Standards and Process Criteria

The Regulation 2073/2005 (EC, 2005) establishes microbiological criteria for *L. monocytogenes* in the category RTE foods able to support growth: (i) *L. monocytogenes* levels should be <100 CFU/g throughout the shelf-life of the product, ii) absence in 25g of the product at the stage before the food has left the immediate control of the food business operator, if the previous criterion has not been satisfied.

Inactivation of pathogens during maturation/storage is, in addition to the control of growth and initial pathogen levels, a crucial step in the safe production of fermented sausages not undergoing heat treatment (Encinas *et al.*, 1999; Drosinos *et al.*, 2005 ;2005; 2007; Porto-Fett *et al.*, 2008; Montet *et al.*, 2009; Lindqvist and Lindblad, 2009), though the potential presence in the product at the time of consumption, still remains a major concern (Thévenot *et al.*, 2005a,b; Nightingale *et al.*, 2006; *et al.*, 2009).

3.4.1 Establishment of "in-house" Performance Objectives to achieve the Food Safety Objective for *L. monocytogenes* in fermented dry meat products

Evaluations of putative risk management actions are often based on comparisons of a baseline risk estimate with a forecast risk that could result from pursuing various alternative strategies (FAO/WHO, 2009). This risk assessment model can be used to estimate the likely impact of intervention strategies by changing one or

more input parameters and measuring the change in the model outputs (Ross *et al.*, 2009a, b; Mataragas *et al.*, 2010). Schothorst *et al.*, (2009) states that the Establishment of Performance Objectives (POs) provides the industry with quantitative targets to be met. The following scenarios are intended to simulate the consequence of a putative regulatory policy (i.e., a possible intervention strategy). Additional control measures, e.g. control of initial levels, can be necessary as well (Lindqvist and Lindblad, 2009; Nauta *et al.*, 2009).

A set of simulations was done, setting maximum concentrations of *L. monocytogenes* in contaminated product at pre-retail point, to levels 0.04 CFU/g (limit of detection), 1.0 CFU/g and 10 CFU/g, by truncating the input distribution (Table 3.14).

Table 3.14 Comparison of risk of listeriosis with different Performance Objectives.

Performance Objective		**Predicted nº of listeriosis cases per year**		
		Intermediate age	**Elderly**	**Perinatal**
"Normal"	**CP1**	0.10861	0.16703	1.14650
	CP2	0.12974	0.19953	1.36953
N_i<10 CFU/g	**CP1**	0.06951	0.10690	0.73376
	CP2	0.09672	0.14875	1.02101
N_i<1 CFU/g	**CP1**	0.03453	0.06096	0.41846
	CP2	0.05400	0.08304	0.57000
N_i<0.04 CFU/g	**CP1**	0.02517	0.03871	0.26575
	CP2	0.05377	0.08269	0.56763

The adoption of a Performance Objective of 10 CFU/g, 1 CFU/g and 0.04 CFU/g reduced the risk of listeriosis for all population sub-groups, at the end of shelf life, by 25,5%, 58.4% and 58.6%, respectively.

Because this strategy controls the maximum concentration, but does not influence the prevalence, it was possible to reduce to a low risk of listerioris to the populations considered. Reduction of initial pathogen concentration has been very effective in reducing the risk of listeriosis in RTE salmon (Garrido *et al.*, 2010). Fels-Klerx *et al.*, (2008) demonstrated how Performance Objectives (POs) for *Salmonella* at various points in the broiler supply chain could be estimated and that the model estimations may support policy-makers in their decision-making process with regard to microbiological food safety. Costs of the measures should also be incorporated in order to decide on their cost-effectiveness.

3.4.2 Use of a bioprotective culture as a preventive measure

Traditional fermented meat products like "Salpicão" and "Chouriça de Vinhais" have a "natural" quality perceived by the consumer, so the use of preservatives or some kind of food processing would "denaturalize" the product. The use of a bioprotective culture that achieves inactivation of pathogens has been gaining increased interest by researchers and industry (Ceylan and Fung, 2000, Drosinos *et al.*, 2005, Leroy *et al.*, 2006; Albano *et al.*, 2007a,b; Lindqvist and Lindblad, 2009). Lactic acid bacteria that produce bacteriocins with antilisterial activity have been used in fermented sausages as both starter cultures and bioprotective cultures (Drosinos *et al.*, 2005, Albano *et al.*, 2007a,b).

3.4.2.1 Screening for bacteriocin-producing lactic acid bacteria

Four hundred and sixty six LAB strains, isolated from various ‘‘Salpicões’’ and “Chouriças”, (Table 3.15) were screened for their antagonistic activity against *L. innocua, E. coli* 0157:H7, *St. aureus, E. coli, Ec. faecalis, S.* Typhimurium and *S.* Enteriditis.

Table 3.15- Sources and media of isolation of LAB strains

Media / **Product**	**MRS** isolates	**M17** isolates
“Salpicão”	67	102
“Chouriça”	142	154
Total	209	256

One hundred and seventeen strains showed antagonistic activity against *L. innocua* and seventy five isolates also showed antagonistic activity against *Ec. faecalis;* these strains were isolated in M17 medium. Antimicrobial activity against the Gram-negative bacteria was not shown by any of the strains tested. Using this screening method, the observation of an inhibition zone, may result from competition, lactic acid, bacteriocin or hydrogen peroxide production. The positive inhibitory effect of the cell-free filtrates of each of the one hundred and ninety two isolates was evaluated. After filtration and neutralization, forty isolates maintained inhibition over *L. innocua*; all isolates lost inhibitory effect over *Ec. faecalis*. After treatment with catalase, twenty one isolates (4.7% of the strains isolated from “Salpicão” and 4.4% of the strains of “Chouriça”), maintained inhibition over *L. innocua* but lost it after treatment with trypsin, indicating the

possibility of antilisterial activity being of proteinaceous nature. It should be noted that, in this study, *L. innocua* was used as a target microrganism. Previous studies have reported a higher sensitivity of *L. monocytogenes* towards some antibacterial compounds than *L. innocua* (Mataragas *et al.*, 2003), thus, the results will be conservative, portraying a "worst case scenario". Also, *L. innocua,* was considered an adequate surrogate for *L. monocytogenes* by Vaz-Velho *et al.*, (1998), Friedly *et al.*, (2008) and NACMCF (2009).

These isolates were tested against several strains (I-V) of *L. monocytogenes*, with different serovars (1/2a, 1/2b, 4a, 4b, 4c).

Table 3.16- Inhibitory activity of LAB against *L. monocytogenes*

Strains	**I** (Sv ½a)	**II** (Sv ½b)	**III** (Sv 4a)	**IV** (Sv 4b)	**V** (Sv 4c)
"Salpicão"	0 (0%)	8 (100 %)	7 (87.5 %)	8 (100 %)	0 (0%)
"Chouriça"	2 (15.4%)	13 (100%)	12 (92.3%)	13 (100%)	0 (0%)

It is interesting to notice that all of the isolates were inhibitory towards serotypes 1/2b and 4b, which have been associated with many sporadic cases of listeriosis in the USA and almost all outbreaks registered in Europe (4b) (Jay, 1996; Kathariou, 2002), and isolated from Italian salami (Gianfranceschi *et al.*, 2009) and frankfurters (Cesar *et al.*, 2011), but were not very effective against serotype 1/2a which is often recovered from raw pork meat (Hof *et al*., 1992), has been responsible for sporadic cases of listeriosis in the USA. (De Cesare *et al*., 2007), and recovered from Spanish meat products (Garrido *et al*., 2009).

3.4.2.2 Identification of the putatively bacteriocin-producing lactic acid bacteria

Gram positive, catalase negative and oxidase negative strains showing anti-listerial activity of a proteinaceous nature were then characterized by physiological and biochemical tests: gas production from glucose, growth at 10 °C and 45 °C, pH 9.6 and with 6.5 % salt and esculin hydrolysis (Table 3.17).

Table 3.17 Physiological and biochemical tests used for the identification of LAB isolated from "Salpicão" and "Chouriça".

Morphology	**CO_2 from glucose**	**Growth at 10 ºC**	**Growth at 45 ºC**	**Preliminary identification**
Cocci	90.5%	100%	95.2%	
Coccobacilli				*Enterococcus* spp
Growth with 6.5%NaCl	**Growth at pH 4.4**	**Growth at pH 9.6**	**Esculin hydrolysis**	
95.2%	100%	95.2%	100%	

A total of twenty one isolates potentially bacteriocin producers were tested. The percentage indicates the relative number of positive strains in each test.

Isolates that grew at temperatures of 10 ºC and 45 ºC, in the presence of 6.5% (w/v) of NaCl and at pH 9.6 and hydrolyzed aesculin, were tentatively classified as members of the genus *Enterococcus*. According to some authors (Collins *et al*., 1984; Devriese *et al.*, 1990; Murray, 1990; Martínez-Murcia and Collins, 1991;

Devriese *et al*., 1993; Svec *et al*., 2001; Vancanneyt *et al*., 2004) a few species of enterococci may not grow in one of these conditions, but all grow at pH 9.6.

3.4.2.3 Genus-specific PCR

A PCR product of 112 bp was obtained for all the isolates (Fig. 3.5), confirming that all of these isolates belong to the genus *Enterococcus*.

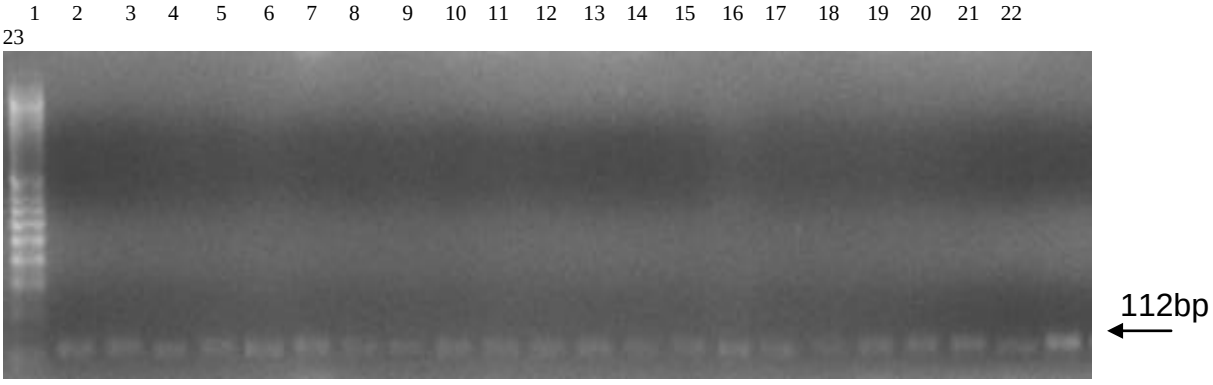


Figure 3.6. PCR products in agarose gel for genus-specific PCR.
Lanes: **1**, molecular weight marker; **2**, to **22**, enterococcal isolates, **23** *Ec. faecalis* ATCC 29212.

Enterococci are sometimes associated with fermented meat products, in particular artisanal products from Southern Europe, where they increase during early fermentation stages and can be detected in the end-products at levels of 10^2 to 10^5 CFU/g (Samelis and Metaxopoulos, 1999; Hugas *et al*., 2003; Franz *et al*., 2003; Papamanoli *et al.*, 2003; Villani *et al.*, 2007, Di Cagno *et al.*, 2008). Though several benefits have been attributed to them, such as their contribution to ripening and aroma development (Rantsiou *et al.*, 2005), probiotic properties and the production of antimicrobial substances, their presence in food products is

considered to be an indication of poor hygienic conditions during processing and handling of food products (Giraffa, 2002; Garcia-Fontan *et al.,* 2007). Opinions diverge about demonstrated beneficial effects such as antimicrobial and probiotic agents and contributing to the sensorial quality, but also affect safety if they are pathogenic strains, causing nosocomial and opportunistic infections (Franz *et al.,* 1999, 2001, 2003; Cocconcelli *et al.,* 2003; De Vuyst, 2000).

The ability of enterococci to produce bacteriocins is well known (Callewaert *et al.,* 2000, Ananou *et al.,* 2005; Sparo *et al.,* 2008). However, intrinsic and acquired antibiotic resistance, the possible presence of virulence factors and their association with human disease can explain their potential pathogenic activity (Jett *et al*., 1994; Stiles and Holzapfel, 1997; Franz *et al*., 2001, 2003; Manero *et al* l, 1999; Giraffa *et al*, 2002, Sparo *et al*, 2008; Gomes *et al*, 2010), so these traits have to be evaluated if they could be considered as a potential bioprotective culture.

3.4.2.4 Antibiotic resistance

An important cause of concern about enterococci is their resistance to a wide variety of antibiotics (Leclercq and Courvalin, 1997; Murray,, 2000; Mathur and Singh, 2005: Ananou *et al.,* 2005, Sparo *et al.,* 2008, Gomes *et al.,* 2010). The MIC (μg/mL), the lowest concentration without visible growth, was determined and the MIC value distributions of the 21 enterococcal isolates are presented in Table 3.18. Clear definitions of breakpoint values that discriminate between resistant and susceptible strains are necessary. Such defined breakpoints should be

considered as a pragmatic response for the separation of suitable and non-suitable strains. Based on MICs, isolates were classified as resistant, intermediary and sensitive to each of the antibiotics investigated, according to NCCLS guidelines (2004; Table 3.18).

Table 3.18 In vitro susceptibility to several antibiotics of enterococcal isolates with anti-listerial activity and minimum inhibitory concentration (MIC) breakpoints)

Antibiotic	**MIC (μg/mL) breakpoints**			**Isolates**		
	S (Sensitive)	**I (Intermediary)**	**R (Resistant)**	**Percentage of sensitive isolates**	**Percentage of intermediate isolates**	**Percentage of resistant isolates**
ampicillin	≤ 8	-	≥ 16	-	-	100
penicillin	≤ 8	-	≥ 16	-	-	100
ciprofloxacin	≤ 1	2	≥ 4	-	5	95
chloramphenicol	≤ 8	16	≥ 32	71.4	28.6	-
erythromycin	≤ 0.5	1-4	≥ 8	-	100	-
nitrofurantoin	≤ 32	64	≥128	4.75	4.75	90
rifampicin	≤ 1	2	≥ 4	-	5	95
tetracycline	≤ 4	8	≥ 16	95	-	5
vancomycin	≤ 4	8-16	≥ 32	100	-	-

Antibiotic resistant enterococci may act as reservoirs that spread antibiotic resistance genes to virulent enterococci and to other pathogenic or opportunistic

bacteria (Giraffa, 2002). Vancomycin resistant enterococci have emerged in the last decade as a frequent cause of nosocomial infections (Rice,, 2001). None of the studied isolates were resistant to vancomycin, which agrees well with results from Barbosa *et al.* (2009). Regarding resistance to β-lactam antibiotics, all isolates were resistant to penicillin and ampicillin. 71.4% of the isolates were sensitive to chloramphenicol, though this antibiotic is seldom used in human treatment and the use in animal husbandry was banned in Europe in 1994 (Peters *et al.*, 2003). A high percentage of the isolates under study showed resistance to ciprofloxacin, nitrofurantoin and tetracycline. Resistance of *Ec. faecium* to ampicillin, tetracyclines, macrolides, aminoglycosides, chloramphenicol, trimethoprim/sulfamethozaxole, quinolones, and streptogramins resistance and related species, was reported by Foulquié Moreno *et al.* (2006). Recent studies with enterococci found resistances to several antibiotics, such as tetracycline, rifampicin, erythromycin, ciprofloxacin, nitrofurantoin and chloramphenicol, as well as sensitivity to ampicillin and penicillin (McGowan-Spicer *et al.*, 2008, Valenzuela *et al.,* 2008, 2009). The ability of enterococci to resist some antibiotics raises a public health concern and thus limits their wide application in the food industry. The rise in enterococcal infections has, in part, been due to the increased use of broad-spectrum antibiotics, both as preventive and therapeutic tools, in human and veterinary applications (Appelbaum, 2000, Giraffa, 2002).

3.4.2.5 Screening-test for production of biogenic amines

Biogenic amines are considered food hazards because of their toxicological effect on consumers (Hugas *et al.*, 2003). The most relevant biogenic amines of bacterial origin in foods of meat origin are tyramine and histamine. Thirteen, of the twenty one putatively bacteriogenic isolates, presented tyramine production and none produced histamine. Tyramine production is an undesirable trait in putative bioprotective bacteria (Sparo *et al.*, 2008). Several authors (Bover-Cid *et al.*, 2001; Ansorena *et al.*, 2002) report the presence of tyramine in fermented sausages, enterococci being one of the groups responsible for its production. Drosinos *et al.*, (2007) reported decarboxylase activity in several LAB and staphylococci strains isolated from traditionally fermented sausages in Southern Greece.

3.4.2.6 Virulence factors

3.4.2.6.1 Presence of virulence genes and its phenotypic expression

To cause infection, enterococci must possess virulence factors which allow the infecting strains to colonize and invade host tissue and move through epithelial cells escaping the host's immune response (Johnson, 1994). The presence of the surface adhesin genes (*efaA*fs, *efaA*fm and *esp*), the aggregation protein gene (*agg*), the cytolysin genes (*cyl*M, *cyl*B, *cyl*A, *cyl*L_L *cyl*L_S) and gelatinase gene (*gel*E) were studied by PCR.

The results showed that 10 isolates (47.6%) possessed the gelatinase gene, but none expressed gelatinase activity. A study by Macovei *et al.* (2009) indicates

that *gel*E expression may induce inflammatory processes in the host. Some authors (Eaton *et al.*, 2001; Franz *et al.*, 2001) demonstrated that there is an elevated prevalence of gelatinase production among *Ec. faecalis* strains isolated from food samples and even when a negative phenotype is found, the strain may possess the relevant gene.

A single isolate (4.8% of the total) presented all cytolysin genes and gene *Eff*Afs, a surface adhesion gene. This isolate expressed the genes, being the only isolate to express it and cause haemolysis. None of the 21 isolates presented the gene for aggregation substance or the other surface adhesion genes, except for the one mentioned above. None of the isolates expressed the enzymes lipase nor DNAase. In general, none of the isolates expressed phenotypically its virulent traits, even if they were present genotypically.

3.4.2.6.2 Biofilm assay

Biofilm production in polystyrene microplates in batch and in fed-batch mode was evaluated (Yi *et al*., 2004). For classification of isolates according to their ability to form biofilms, a cut-off value (ODc) was obtained (Yi *et al.*, 2004). The average OD for all negative controls was 0.032, so the ODc was considered 0.063. Therefore, the strains were classified as non-biofilm producers ($OD \leq 0.064$), weak biofilm producers ($0.080 < OD \leq 0.128$), moderate biofilm producers ($0.128 < OD \leq 0.256$) and strong biofilm producers ($OD > 0.256$) for each assay: batch and fed-batch (Stepanović *et al*., 2000).

In batch mode, all 21 isolates were classified as weak biofilm producers. In fed-batch mode, 61.9%, 33.3% and 4.8% were classified as weak, moderate and strong biofilm producers, respectively. Biofilms may also contribute to pathogenicity of enterococci since bacterial adherence to host tissue is a crucial step in infectious processes (Gomes *et al.*, 2010).

3.4.2.7 Discussing the use of *Pediococcus. acidilactici* HA-6111-2 as a bioprotective culture.

The results obtained like antibiotic resistance, the potential for tyramine production, the presence of virulence genes and biofilm production ability, along with the reservations presented by a significant part of the scientific community (Franz *et al.*, 1999, 2003, Pimentel *et al.*, 2007, Gomes *et al.*, 2010) as well as part of the industry, regarding the use of enterococci as an added fermentative culture, lead to the conclusion that none of the presumptive bacteriocinogenic strains isolated previously was suitable for further study. It was considered more interesting to study further a bacteriocinogenic strain *Pediococcus acidilactici* HA6111-2, isolated from "alheira", which is a meat product produced with very similar microbial ecology, already thoroughly tested and characterized (Albano *et al.*, 2007,a 2007b) and apply it in a different study, and in a different food matrix. The aim of analysing the effect of using a bioprotective culture, in the scope of the risk assessment, would be equally achieved with an already thoroughly tested and characterized, potential bioprotective culture like *Ped. acidilactici* HA-6111-2, that would continue to be studied.

3.4.3 Evaluation of *Pediococcus acidilactici* HA-6111-2 as a biopreservative culture for "Salpicão" and "Chouriça de Vinhais"

The purpose of the work previously described was to obtain a putative bacteriocinogenic LAB strain that could be used as a biocontrol strategy against *L. monocytogenes* in traditional dry fermented smoked sausage. The following sections intend to evaluate and mathematically describe this process, and use it, subsequently, in an alternative scenario of the Quantitative Microbiological Risk Assessment, in order to reduce risk of illness.

This study was partially performed in the pilot plant of Escola Superior de Biotecnologia where it is not possible to work with pathogenic organisms. As previously discussed, *L. innocua* is a suitable surrogate for *L. monocytogenes* towards some antibacterial compounds.

Numerous studies have confirmed the usefulness of bacteriocinogenic LAB in the production of fermented sausages, based on enhanced reduction of inoculated pathogens e.g. *L. monocytogenes* (Mattila *et al.*, 2003; Benkerroum *et al.*, 2003; Drosinos *et al.*, 2005, Albano *et al.*, 2007a,b, Albano *et al.*, 2009).

During the challenge testing performed, the LAB population, autochthonous or added, increased rapidly during early fermentation (an increase of 1.5 to 3.5 log CFU/g) in all samples, dominating the microflora around 8 log CFU/g, which agrees with literature values (Cenci-Goga *et al.*, 2008, Fernandez-Lopez *et al.*, 2008, Zdolec *et al.*, 2008; Porto-Fett *et al.*, 2010).

The initial pH values of the batters were similar (ranging from 5.4-5.5) and the final pH variation ranged slightly from 0.01 to 0.30 in both samples with autochthonous LAB as in the ones with added bacteriocin positive culture. The experiment was ended when the a_w was lower than 0.90, i.e. the minimum water activity to be considered a dry fermented meat sausage, according to Lücke (1998). Since samples were taken weekly, final a_w values in "Chouriça" were 0.1-0.3 units lower than for "Salpicão", probably because of their smaller calibre.

Three scenarios (or treatments) were considered: LI, which corresponds to a regular batch, that is contaminated with *Listeria* spp. and undergoes the process of smoking ; LI+LABneg, which mimics a batch contaminated with *Listeria* spp. and with an added starter culture with no bacteriocinogenic activity, simulating the competition effect between bacterial groups; and LI+LABpos, that corresponds to a batch, contaminated with *Listeria* spp and to which a bioprotective culture that produces a bacteriocin (see 3.4.2), was added.

In Fig. 3.7 and Fig. 3.8 it can be observed a slow decrease in the counts of *L. innocua*, by 1 log during 5 weeks of smoking process.

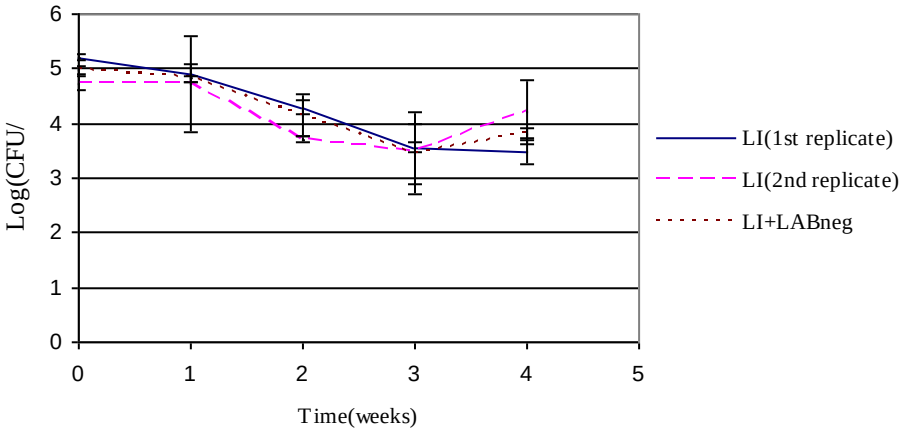


Fig. 3.7 Comparing the effect of smoking *on L. innocua* with the negative LAB used as control, for "Salpicão de Vinhais". (LI - *L. innocua*, LI+LABneg - *L. innocua* + non-bacteriocinogenic LAB strain).

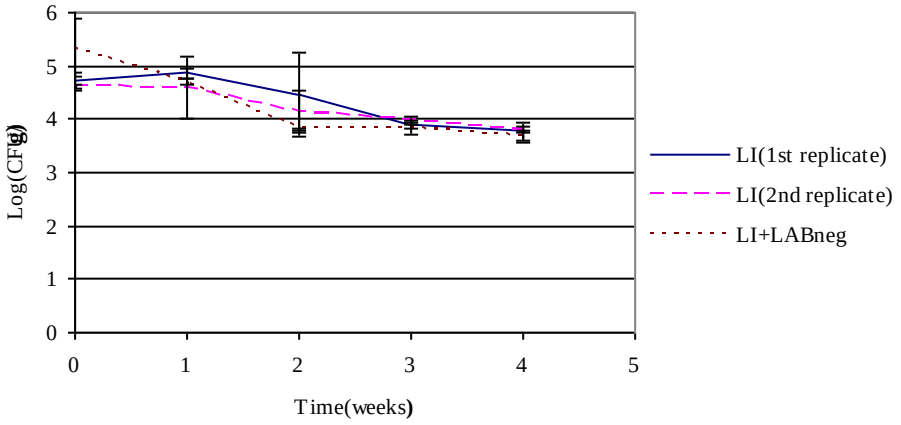


Fig. 3.8 Comparing the effect of smoking on *L. innocua* with the negative LAB used as control, for "Chouriça de Vinhais" (LI - *L. innocua*, LI+LABneg - *L. innocua* + non- bacteriocinogenic LAB strain).

Several studies have reported the hurdle effect (Tyopponnen *et al.*, 2003; Arnau *et al.*, 2007; Thomas *et al.*, 2008), occurring during the smoking process as argued before (see 3.2.1). The slight decrease in pH is not the main factor responsible for pathogen control since there is a buffer effect in these products as LAB grow, with concomitant production of organic acids, such as lactic acid (Fernandez-

Lopez *et al.*, 2008). Another possible explanation may be the action of yeasts (contributed by the raw meat) which use lactic acid as a substrate at the end of the drying step (Thévenot *et al.*,, 2005 b). The reduction of *Salmonella* spp., *Listeria* spp., *St.aureus* and anaerobic sporeformers, due to the LAB growth during fermentation of low acid fermented sausage, has been studied previously (Cenci-Coga *et al.*,, 2008) . The fermentation and drying of French fermented meat sausages caused different effects on acid-resistant and non-acid resistant non-O157 *E. coli* (Montet *et al.*,, 2009). Porto-Fett *et al.*, (2008) concluded that conditions in soudjouk-style sausages do not provide a favorable environment for outgrowth/survival of these and described the effect of fermentation, drying, and storage on *L. monocytogenes, S.* Typhimurium and *E. coli* O157:H7.

Statistical comparison:

The two way ANOVA indicates that there are no differences between the results for counts of *L. innocua* for both replicates, for each product, ($F_{replicate}$ 2.85, $P \geq 0.05$) of the challenge testing performed, so they can be grouped in posterior analysis. The same statistical testing reveals that the inoculation of a LAB negative-producing for bacteriocin, has no different effect on *L. innocua* than the simple smoking process does, for both products "Salpicão" and "Chouriça", ($F_{product}$ 2.07; $P \geq 0.05$).

Figures 3.8 and 3.9 show that *Ped. acidilactici* HA-6111-2 had a marked antagonistic effect on the growth of the cocktail of *L. innocua* in both "Salpicão" and in "Chouriça" , respectively, during the smoking process. The two way

ANOVA indicates that there were no differences between the results for counts of *L. innocua* between "Salpicão" and "Chouriça", ($F_{product}$= 4; P≥0.05), but there was a statistically significant difference between batches submitted to a simple smoking process (LI) only, and with the addition of a bacteriocinogenic LAB (LI+LABpos) ($F_{treatment}$=15.17; P<0.05). This means that "Salpicão" and "Chouriça" behave similarly to both treatments.

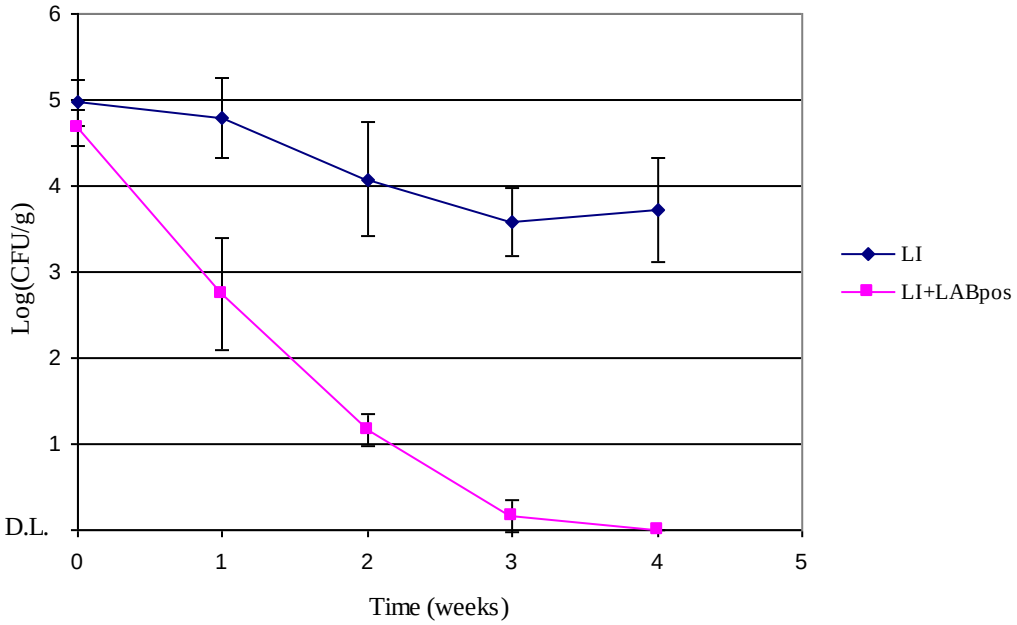


Fig. 3.9 Non-thermal inactivation of *L. innocua* by *Ped. acidilactici* HA-6111-2, during smoking step of "Salpicão de Vinhais" (LI - *L. innocua*, LI+LABpos - *L. innocua* + bacteriocinogenic LAB strain); D.L.-Detection limit.

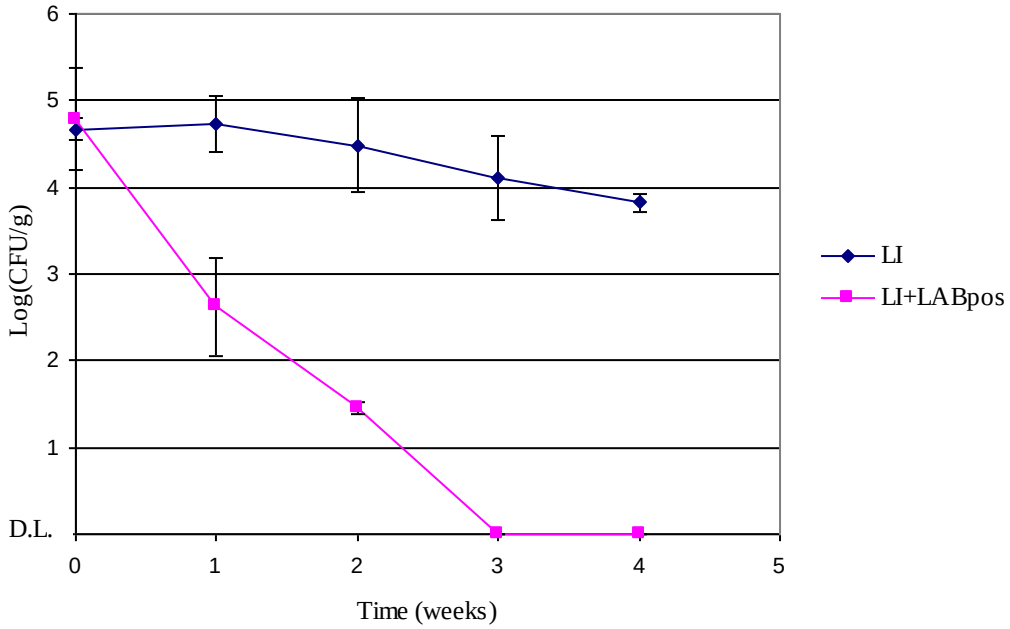


Fig.3.10 Non-thermal inactivation of *L.innocua* by *Ped. acidilactici* HA-6111-2, during smoking step of "Chouriça de Vinhais" (LI - *L. innocua*, LI+LABpos - *L. innocua* + bacteriocinogenic LAB strain); D.L.-Detection limit.

Also, the addition of the bacteriocinogenic strain, HA6111-2 was effective in reducing *L. innocua* counts, and presumably, also in reducing *L. monocytogenes*, for both of these products. According to Lücke (2000), the *in situ* effect of bacteriocins against *L. monocytogenes* in meat systems has resulted, in most reported cases, in the reduction of *L. monocytogenes* counts by 1–2 log units compared with a bacteriocin-negative control. Benkerroum *et al.*, (2003) reported a reduction of 1.52 log units in the Bac positive samples compared with the Bac negative control samples. Callewaert *et al.*, (2000) reported a decrease in *Listeria* count by 1.65 log CFU/g, with sakacin K-producing *Lb. sakei* CTC 494 strain, with the Bac negative strains *Ec. faecium* RZS C13 and the *Ec. faecium* CCM 4231 obtaining more significant reductions (3.28 and 3.25 log, respectively).

These values are considerably less than the results obtained with strain HA6111-2, which achieved a reduction of 3.7-3.8 log units.

Porto-Fett *et al.*, (2008) reported that fermentation and drying alone reduced numbers of *L. monocytogenes* by 0.07 and 0.74 log10 CFU/g in soudjouk sausages. Nightingale *et al.*, (2006) reported slight growth of *L. monocytogenes* during drying of Italian-style salami. Johnson *et al.* (1988) reported the survival of *L. monocytogenes* during the fermentation and drying of salami when initial populations were higher than 3.0 log CFU/g.

Unlike some semidry sausages, dry fermented sausages, usually, are not exposed to pathogen inactivating temperatures. Consequently, dry sausages have attracted more attention, as shown by several studies that examined the fate of *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* during the fermentation, slicing and storage of hard salami, pepperoni and several other sausages (Lahti *et al.*, 2001; Barbuti and Parolari, 2002; Chikthimmah *et al.*, 2001; Hew *et al.*, 2005; Cenci-Goga *et al.*,, 2008, Porto-Fett *et al.*,, 2008; Montet *et al.*,, 2009).

3.4.3.1 Modelling the effect of *Ped. acidilactici* HA-6111-2 as a biopreservative culture for "Salpicão" and "Chouriça de Vinhais"

Selection of the best model

The biphasic with shoulder, linear, Geeraerd with shoulder, Geeraerd with tail (Geeraerd *et al*., 2000), Weibull, modified Weibull (Albert and Mafart., 2005), Biphasic, Baranyi, Biphasic logistic and reparameterized Gompertz were fitted to the experimental data of *L. innocua* inactivation. These models were compared by

the calculation of several statistical indices which are presented in Tables 3.19 and 3.20. All models were accepted for LOF (Lack-Of-Fit) with the F test for the three treatments. The criteria for selecting the best fit are higher r^2, lower A_f, MSE_{model} and F statistics. Hence, for the batch in which the only treatment was the smoking process (LI) and the batch with a non-bacteriocinogenic LAB, the best model was biphasic logistic with very similar statistical indices. Not surprisingly, the same model was the one that best fit to both products, "Salpicão" and "Chouriça", since the data were not statistically different (see 3.2.2). Regarding the batch, in which the only treatment was the smoking process (LI) and the batch with added HA-6111-2 to the batter (LABpos), though the reparametrized Gompertz presented a slightly higher r^2 in "Salpicão", the other statistical indices were more favourable for the biphasic model. So, the biphasic equation was the selected model to represent the non-thermal inactivation of *L. innocua*, for both "Salpicão" and "Chouriça", with the three processes considered (effect of smoking only, effect of smoking and LAB competition and effect of smoking and bacteriocinogenic LAB). Drosinos *et al* (2005) reported that in fermented sausages typical of four European countries, inactivation during fermentation and ripening increased in the presence of a bacteriocin producing *Lb. sakei* strain and was best described by the model of Baranyi and Roberts (1994).

Table 3.19 Statistical comparison of the models used to describe the non thermal inactivation of *L. innocua* in "Salpicão de Vinhais"

Treatment	Statistical indices	Models									
		Biphasic w/ shoulder	Linear	Geeraard shoulder	Geeraerd tail	Weibull	Modified Weibull	Biphasic	Baryani	Biphasic logistic	Reparametrized Gompertz
LI	r^2	0.7227	0.5824	0.6369	0.423625	0.6321	0.6321	0.6072	0.7165	**0.74608**	0.7453
	A_f	0.214598	0.235872	0.229345	0.24685	0.230254	0.230254	0.234208	0.212492	**0.204097**	0.205514
	MSE_{model}	0.1389	0.1758	0.1586	0.251681	0.1607	0.1668	0.1781	0.1286	**0.119747**	0.1201
	LOF[(a)]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	**Yes**	Yes
	F	1.5806	1.337145	1.205778	1.913941	1.221741	1.268731	1.354673	0.824427	**0.814167**	0.811653
	F_{table}	2.98	1.93	1.94	1.94	1.94	1.95	1.95	1.95	**1.96**	1.96
LI+LABneg	r^2	0.6971	0.6822	0.6889	0.73324	0.6894	0.6894	0.682186	0.682197	**0.79509**	0.753441
	A_f	0.175192	0.217191	0.215924	0.206158	0.216042	0.216042	0.217191	0.217188	**0.189874**	0.194542
	MSE_{model}	0.0763	0.1484	0.1574	0.135	0.157154	0.1714	0.175395	0.175389	**0.124395**	0.149678
	LOF	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	**Yes**	Yes
	F	1.361335	0.864279	0.91658	0.785894	0.95189	0.998388	1.02142	0.194365	**0.122514**	0.147416
	F_{table}	2.98	2.89	2.91	2.91	2.91	2.94	2.94	2.94	**0.79509**	2.98
LI+LABpos	r^2	0.959	0.8674	0.9125	0.9591	0.9257	0.9598	**0.959**	0.914451	0.911881	0.962642
	A_f	0.425757	0.376966	0.33643	0.45219	0.33489	0.451109	**0.323399**	0.776821	0.424998	0.335353
	MSE_{model}	0.2189	0.6228	0.4281	0.2001	0.3637	0.2051	**0.2193**	0.29665	0.439094	0.221257
	LOF	Yes	Yes	Yes	Yes	Yes	Yes	**Yes**	Yes	Yes	Yes
	F	0.607025	1.727139	1.187221	0.554827	1.00854	0.568814	**0.580518**	0.132603	1.063981	0.498902
	F_{table}	1.96	2.46	2.48	2.48	2.48	2.51	**2.51**	2.51	2.53	2.53

(a) LOF was either accepted (yes) or not accepted (no) based on the *F* test

Table 3.20 Statistical comparison of the models used to describe the non thermal inactivation of *L. innocua* in "Chouriça de Vinhais"

Treatment	Statistical indices	Models									
		Biphasic w/ shoulder	Linear	Geeraard shoulder	Geeraerd tail	Weibull	Albert	Biphasic	Baryani	Biphasic logistic	Reparametrized Gompertz
LI	r^2	0.6585	0.6268	0.6342	0.6268	0.6361	0.6771	0.6268	0.626866	**0.681332**	0.65202
	Af	0.185369	0.190774	0.190855	0.190774	0.190660	0.181209	0.190774	0.190771	**0.179244**	0.187146
	MSE_{model}	0.086	0.0839	0.0853	0.0870	0.0848	0.0782	0.0903	0.090329	**0.080229**	0.087609
	LOF[(a)]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	**Yes**	Yes
	F	0.898509	0.876573	0.891085	0.909038	0.886575	0.816820	0.944001	0.853634	**0.796130**	0.869361
	F table	1.96	1.93	1.94	1.94	1.94	1.95	1.95	1.95	**1.96**	1.96
LI+LABneg	r^2	0.9005	0.7599	0.8435	0.8835	0.8423	0.8423	0.8423	0.371729	**0.90062**	0.764944
	Af	0.155558	0.199427	0.190982	0.173608	0.191521	0.191521	0.191520	0.239343	**0.153495**	0.184831
	MSE_{model}	0.0680	0.1262	0.0891	0.0663	0.0898	0.089754	0.0979	0.390151	**0.067886**	0.160564
	LOF	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	**Yes**	Yes
	F	0.527109	0.978695	0.690808	0.514482	0.696201	0.696201	0.759492	0.398829	**0.043099**	0.164136
	F_{table}	2.98	2.89	2.91	2.91	2.91	2.94	2.94	2.94	**2.98**	2.98
LI+LABpos	r^2	0.8636	0.9013	0.9167	0.9284	0.9205	0.9288	**0.9288**	0.855551	0.858076	0.884143
	Af	0.409959	0.322727	0.328703	0.388871	0.338510	0.382329	**0.27740**	0.289861	0.30394	0.381971
	MSE_{model}	0.7937	0.4838	0.4305	0.3701	0.4112	0.3900	**0.3417**	0.316420	0.418615	0.3900
	LOF	Yes	Yes	Yes	Yes	Yes	Yes	**Yes**	Yes	Yes	Yes
	F	2.322504	1.415620	1.259904	1.082955	1.203208	1.141113	**1.141133**	0.107414	0.132937	0.108521
	F_{table}	2.53	2.46	2.48	2.48	2.48	2.51	**2.51**	2.51	2.53	2.53

(a) LOF was either accepted (yes) or not accepted (no) based on the *F* test

Since the biphasic logistic is not a totally linear equation, presenting a "shoulder", equation (2) is more acceptable. For these batches, the estimated parameter f (the fraction of the initial population in a major subpopulation) was superior to 99%, meaning that the effect of k_1 was dominant and the contribute of k_2 was negligible. The parameter k_2 was considered not significant and only k_1 was used in the t_{4D} calculation,

$$t_{4D} = \frac{\log\left[\frac{1+\exp(-k\times t_s)}{0.0001}-1\right]+k\times t_s}{k} \qquad \text{Eq.2)}$$

The simplest equation (3) for calculation of the time necessary for a 4-log reduction of the pathogen comprises both the lag phase and the inactivation phase and is adequate to use with the biphasic model.

$$t_{4D} = t_s + 4\times D \qquad \text{Eq.3)}$$

Plotting the results of the sampling points of the experiment with the bacteriocinogenic LAB, it appears to have no shoulder and a fairly linear behaviour, so equation (3) was used. The selected models (biphasic logistic or biphasic) were applied to individual replicates of the different experimental trials and the kinetic parameters, k, ts, No and $t_{4D,}$ for each experimental trial was calculated. Table 3.21 summarizes the mean kinetic parameters calculated and respective estimated values of initial contamination N_0 and calculated t_{4D}.

Table 3.21 Estimation of the kinetic parameters of non-thermal inactivation of *L. innocua* according to biphasic-logistic (LI and LI+LABneg) and biphasic (LI+LABpos) models.

Treatment [a)]	**k** (day^{-1})	**t_s** (days)	**N_0** (log CFU/g)		**t_{4D}** (days)	
"Salpicão"						
LI	0.641	6.17	5.21	cd	22.03	a
LI+LABneg	0.538	6.27	5.20	c	23.93	a
LI+LABpos	0.613	0.00	4.95	cd	15.04	b
"Chouriça"						
LI	0.702	6.62	5.16	cd	23.45	a
LI+LABneg	0.687	5.36	5.34	cd	22.25	a
LI+LABpos	0.647	0.00	4.77	d	14.87	b

a) All treatments include a smoking step, simulating the industrial reality.

Statistical Analysis:

i) Comparison between controls (LI and LABneg) and treatment with bacteriocinogenic strain (LABpos), within each product.

The one-way ANOVA and comparison of N_0 and t_{4D} calculated for individual replicates of the different experimental trials, using the Student's t- test were used to evaluate differences between N_0 and t_{4D} for the three treatments, within the same product, "Salpicão" or "Chouriça". Regarding the initial contamination, N_0, the one-way ANOVA for both products indicated statistically significant differences between the treatments. The treatments were then compared two by two, using a t-test, indicating a significant difference between the process with the normal smoking step (LI) and the process with the added bacteriocinogenic HA 6111-2 (LABpos).

Regarding the t_{4D} parameter, a similar analysis indicated, as expected, significant differences between t_{4D} for treatment with bacteriocinogenic HA 6111-2

(LABpos) and the other treatments, within each product. No significant differences were observed between t_{4D} for LI and LABneg. This agrees well with the conclusion that the data were not statiscally different between treatments (see 3.2.2).

ii) Evaluation of the effect of product "Salpicão" and "Chouriça" on the values of N_0 and t_{4D}**.**

Two sources of variation were included in this analysis: the effect of the three treatments (LI, LABneg and LABpos) and the effect of type of product ("Salpicão" or "Chouriça"). For this evaluation, a two-way ANOVA analysis was conducted on the mean values of N_0 and t_{4D} and paired comparison with Student's t-test was performed to evaluate every possible combination of effects. This revealed a significant difference between treatments ($F=8.12$, $P<0.05$) and ($F=29.16$, $P<0.05$) for N_0 and t_{4D}, respectively, but no significant difference between "Salpicão" and "Chouriça" ($F=0.144$, $P\geq0.05$) and ($F=0.022$, $P\geq0.05$), respectively. Also, no significant interaction between those two variables (treatment and product) was found for N_0 and t_{4D}, with ($F=1.18$, $P\geq0.05$) and ($F=0.83$, $P\geq0.05$) respectively. As expected, the smoking process with the added HA 6111-2 (LABpos) treatment was significantly different from the normal smoking process and the smoking process with non-bacteriocinogenic strain added (LABneg). The values of N_0 and t_{4D} in the normal smoking process (LI) and smoking process with non-bacteriocinogenic strain added (LABneg) were not statistically different between "Salpicão" and "Chouriça".

Again, the treatment with HA-6111-2 (LABpos) in "Salpicão" was not statistically different from the same treatment in "Chouriça". This seems to indicate that product calibre ("Salpicão" has a wider diameter than "Chouriça") and batter texture (the same recipes used, the only difference is that whole pieces of pork meat are used in "Salpicão", and more finely cut meat is used in "Chouriça") do not influence the non-thermal inactivation rate in these products, which simplifies its possible application, at plant scale. Again, the use of data from "Salpicão" and "Chouriça" as if it was only one product "Traditional Dry Fermented Smoked Sausage" (see 3.2.2), is validated.

As previously stated, if statistical analysis indicates that there is no difference between the kinetic parameters used in the biphasic-logistic and biphasic models, then the average value of t_{4D} (i.e. considering "Salpicão" and "Chouriça" as a single foodproduct - traditional dry fermented smoked sausage) - can be used in the new calculations in the new exposure assessment, simulating the use of bacteriocinogenic strain HA6111-2 as a Risk Management tool, to lower the risk of listeriosis. The following sections describe the Risk Management scenarios necessary to lower the risk estimate of listeriosis calculated in 3.3.4.

3.4.4 Establishment of "in-house" Performance Criteria to achieve the Performance Objective previously proposed.

This risk assessment indicates that most cases of listeriosis result from consuming high levels of *L. monocytogenes* in food that permits its growth. Interventions might be designed to reduce the number of *Listeria* in food before it is sold. The

L. monocytogenes Risk Assessment, by FDA/FSAN (2003), suggests post-retail interventions, such as refrigerator temperature scenarios, storage time scenarios (limited/extended), storage time and temperature interaction scenario, cooking scenario, disease rate as a function of concentration levels at the time of consumption. It also suggests pre-retail and retail interventions such as reduction of the number of organisms scenarios. Schonning *et al.*, (2007) also evaluated several scenarios to choose best measures to further reduce the risks of transmission of infectious disease related to the local use of faeces as a fertiliser. Ross *et al.*, (2009c) included in the calculation of risk, the effect of LAB, both as a spoilage flora (leading to consumer rejection) and having an inhibitory effect according to the principle of the Jameson effect. Besse *et al.*, (2006) reported an important effect of the background microflora on the maximum population of *L. monocytogenes* growth. Garrido *et al.*, (2010) explored four scenarios to assess the reduction of risk of listeriosis in RTE foods, by reducing home storage time, decreasing refrigerator temperature, combining short time-low temperature storage, limit the pathogen concentration to 100 CFU/g at the moment of food consumption. In all of the products, the scenario that combined storage time with the recommended temperature decrease, storage at low temperature was the most effective, followed by storage at low temperature and, to a lesser extent, reduction of storage time.

The effect of setting possible Process Criteria such as non-thermal inactivation by addition to the batter of bacteriocinogenic HA 6111-2, the water activity of final product below 0.92 and both of these measure combined is resumed in Table 3.22.

Table 3.22 Comparison of risk with different Risk Management scenarios

		Predicted nº of listeriosis cases per year		
		Intermediate age	**Elderly**	**Perinatal**
"Normal"	**CP1**	0.10861	0.16703	1.14650
	CP2	0.12974	0.19953	1.36953
N_i' with HA 6111-2	**CP1**	0.07228	0.11116	0.76296
	CP2	0.09507	0.14621	1.00355
With a_w<0.92	**CP1**	0.00077	0.00118	0.00811
	CP2	0.11854	0.18231	1.25136
N_i' with HA 6111-2	**CP1**	0.00054	0.00084	0.00574
With a_w<0.92	**CP2**	0.07665	0.11788	0.80911

The use of a bacteriocinogenic strain added to the batter reduced by 26.7% the risk of listeriosis for all sub-groups, at the end of shelf life. Obtaining a final product with a_w below 0.92, supposedly below growth limits of *L. monocytogenes*, reduces the risk by 8.6% in all sub-groups of population. Though the pathogen might not have conditions to grow, at a_w inferior to 0.92, it does survive. If the organism is present, it will remain and be able to cause infection. Even with the a_w in the final product below 0.92, some growth of *L. monocytogenes* may occur in the raw ingredients or during the initial phases of the fermentation, particularly if products are not inoculated with a starter culture (Campanini *et al.*, 1993). This could extend the time during which the product composition might allow growth of *L. monocytogenes*. Water activity has been recommended to be monitored and controlled as an important factor regarding the safety of high risk products, in relation to *L. monocytogenes* (Luber *et al.*, 2011). The combination of the bacteriocinogenic strain and the limit of a_w 0.92 reduce the risk by 41.0%, representing the "Hurdle Effect". In the relevant literature this effect is identified

as the ability of all LAB to produce organic acids and decrease the pH of foods in which they grow. Other factors such as the production of bacteriocins, hydrogen peroxide and ethanol may all play a contributory role in assuring the safety of fermented meat products (Chirife, 1993; Adams and Nicolaides, 1997, Holzapfel *et al.*, 2004). Other types of scenarios could be assessed. Perez-Rodrigues *et al.*, (2006) evaluated the contamination of hands by contact with minced chicken meat contaminated with *L. monocytogenes* (wearing or not wearing gloves and washing or not washing hands) and subsequent cross-contamination from these contaminated hands to a cooked ham slice (including or not including the use of gloves and hand washing) and used the exposure assessment model to prioritize the risk and propose appropriate risk management strategies

CHAPTER 4. CONCLUSIONS

There is little information regarding many traditional food products, in which Portugal is so rich, though they have considerable economical and cultural value. The structure of the production system, consisting of small scale units, with low investment finance, is an hindrance to the performance of research. This study was undertaken with the aim of developing a quantitative risk assessment model to provide a prediction of listeriosis cases by consumption of contaminated RTE products and to assess the effects of the application of control measures to reduce illness (i.e. risk management).

In this case, focusing on the microbiological problem of traditional Portuguese dry fermented smoked sausages, "Salpicão de Vinhais" and "Chouriça de Vinhais", has been useful for obtaining more realistic data, for identifying the gaps or limited information and to explore some scenarios that the local producers may choose to explore.

- *Listeria monocytogenes* was the pathogen of concern in these products; also noteworthy is the fact that the microbiological and chemical characteristics of "Salpicão" and "Chouriça de Vinhais" are not statistically different.

- These results contradict the general assumption that dry fermented smoked sausages are unable to support the growth of *L.monocytogenes* (the pathogen of concern), according to the Commission Regulation (EC) 2073/2005. This reflects the variability associated with the production process. This knowledge should benefit the small scale producer and allow them to take measures that contribute

to product safety. The use of predictive microbiology models is a simple and low cost tool to help producers to demonstrate compliance with the Regulation criteria.

- If some units of traditional dry fermented smoked sausages are able to support *L. monocytogenes* growth, while others do not, the question of "What is the risk of listeriosis?" due to these products, becomes a more relevant issue.

- Even if a considerable percentage of the products do not comply with legal criteria, as many of the samples tested fall within boundary growth conditions for *L. monocytogenes*, the result of the baseline Quantitative Microbial Risk Assessment, indicated that, for the Portuguese population, Traditional Dry Fermented Smoked Sausages constitute a low risk for the Intermediate Age and Elderly population and an intermediate risk for the Perinatal populations. This is noteworthy information for pregnant women, to reduce the consumption of these products as a precaution.

- Though this estimate is favourable to the Industry, it is affected by a high variability. It should be noted that this risk estimate is highly influenced by the variability and uncertainty of parameters and by the type of model that was built. It should not prevent producers from implementing measures to further increase the safety of their products.

- The Quantitative Microbial Risk Assessment model was used as baseline to evaluate the effectiveness of different risk management options or control measures (i.e. "what-if" scenarios). Model building is a laborious task but after it is available, it rapidly provides insights regarding the effectiveness of possible

options to be undertaken, at Governmental decision level or at Industry food safety level. Nevertheless, outcomes need not be taken for granted, but should always be supported by expert knowledge, literature data, competing models and experiments.

- It was possible to evaluate the effectiveness of risk management decisions such as setting Performance Objectives, defining a limit for the concentration of *L. monocytogenes* in the final product of 10 CFU/g, 1 CFU/g and 0.04 CFU/g. Though, at first sight, the more stringent, the better, results indicate that the lower PO value would be inadequate as it does not reduce significantly the risk of listeriosis and would, presumably, be more difficult and expensive to achieve by the producers.

- In this work, it was demonstrated that the possible use of a bioprotective culture such as *Ped. acidilactici* HA-6111-2, isolated from another traditional fermented smoked sausage, and a pediocin PA-1 producer, resulted in a significant reduction of risk for the entire population. This would be a more "natural" measure, avoiding synthetic preservatives and maintaining the "traditional" and "additive-free" characteristic of these products.

- Another simple measure such as the monitoring of water activity during processing, assuring that the final product reaches an a_w equal or lower than 0.92, also reduces risk significantly. Here is an example where a small investment in a monitoring equipment, translates quantitatively in an increase in product safety, reducing (but not eliminating) the need for extensive microbiological testing.

As far as the author's knowledge, this type of work has not yet been performed, in Portugal, where this subject is still an incipient object of research. This work has shown the usefulness of quantitative microbiological risk assessment as a tool for protecting Public Health by Government authorities, but also for improving food safety by the private sector, by assessing different strategies to reduce risk or contamination at a given point in the production/distribution chain. It has also underlined several data gaps and need for other information, and a rigorous critical review of results, in order to obtain an accurate estimate. Risk assessment is only as good as the data, the model used and the critical judgement of the results. The quantitative microbial risk assessment, or parts of it, may be used to guide Government Regulatory decisions and to evaluate the cost/effectiveness of possible management measures. The usefulness of bioprotective cultures as a "natural" way to reduce contamination in a Traditional Fermented Smoked Sausage was, once again, tested with success.

CHAPTER 5. PROPOSALS FOR FUTURE WORK

The following suggestions for future work can be made:

- There is a clear need for additional quantitative data on *L. monocytogenes* contamination, regarding both concentration and prevalence. The number of contaminated samples detected experimentally are detectable contaminations arising from a continuous log normal distribution of contamination, and the minimum detectable level from the presence/absence tests is typically 1 organism in 25 g or 0.04 organisms per gram. A low percentage of samples are contaminated at or above this level with the remaining samples having non-detectable levels (i.e., lower than 0.04 organisms per gram). This results in a high level of uncertainty in the probability distribution of one of the most influential variables for the Quantitative Microbial Risk Assessment;
- Extend the model of quantitative microbial risk assessment developed to other traditional fermented smoked sausages, e.g. “Alheiras”;
- The study could differentiate between small-scale producers and industrial producers. Typically, the latter presented higher water activity values;
- The model was constructed based on knowledge concerning consumer habits regarding “chouriço” products, which include a wide variety of meat products, with very different characteristics and prices, which would probably affect consumption patterns. It has been assumed that the whole population follows the described distribution of serving size and frequency of consumption per year, but the fact that an unknown percentage of the population could have

different habits of consumption, must be kept in mind. Continuous effort should be made to obtain more data for future improvement of the model;

- An important lack of data has been found in the dose-response model, due to the assumption that all *L. monocytogenes* strains have a similar virulence, when it is not so. Introducing the virulence of a strain in the model would be an interesting challenge;
- Taking into account the difficulty in carrying experiments to obtain dose-response data, this is a current assumption in several risk assessments, that could be improved;
- To assess the efficiency of intervention measures, by additionally incorporating costs of intervention measures and other (societal) aspects that may influence the risk manager's decision;
- Reduce uncertainty of parameters, evaluate the model uncertainty, exploring novel and advanced statistical tools; completing the model evaluation by perfoming an adequate model validation;
- Explore alternative mitigating strategies such as high pressure pasteurization, by performing challenge testing, at pilot scale.
- Test the sensory acceptability of “Salpicão” and “Chouriça de Vinhais” produced with the addition of the bioprotective culture or treated by high pressure pasteurization;

CHAPTER 6. REFERENCES

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