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UNIVERSIDADE CATÓLICA PORTUGUESA | PORTO

Escola Superior de Biotecnologia

Selection of an antimicrobial culture to be used in the prevention of neonatal listeriosis

Thesis submitted to the Universidade Católica Portuguesa to attain the degree of PhD in Biotechnology - with specialization in Microbiology

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Sandra Cristina Ferreira Borges

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Under the supervision of Professor Paula Cristina Maia Teixeira

Under the co-supervision of Dr. Joana Gabriela Laranjeira Silva

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Abstract

The capacity of lactic acid bacteria to produce acidic products and/or secrete antimicrobial compounds is important in the impairment of vaginal colonization by pathogens. Vaginal pH is normally acidic, varying between 3.5-4.5; an increase in vaginal pH (5.0 to 6.5) can be associated with colonization by pathogenic microorganisms.

The main goal of this study was to select an antimicrobial culture to be used in the prevention of vaginal colonization of *Listeria monocytogenes* during pregnancy, and consequently, prevent neonatal listeriosis.

The survival and biofilm formation of 20 isolates of *L. monocytogenes* in simulated vaginal fluid at normal vaginal pH (4.2) and at higher pH values (5.5 and 6.5) was investigated. This pathogen was inhibited by the normal vaginal pH but survives when pH increases. All isolates tested were biofilm producers at different pH values.

Streptococcus agalactiae is an important cause of neonatal infection and maternal colonization. Therefore, its behavior in simulated vaginal fluid was also analyzed. As with *L. monocytogenes, S. agalactiae* (n=10) survived longer at higher pH values than at normal vaginal pH. All *S. agalactiae* isolates were also biofilm producers.

Therefore, since *L. monocytogenes* and *S. agalactiae* can survive at higher vaginal pHs, fetuses/neonates from women having increased vaginal pH values during pregnancy, may be at higher risk of neonatal infection. Biofilm production increases the probability of occurrence of neonatal infection.

The application of vaginal probiotics could have the potential for preventing vaginal *Listeria* colonization in pregnant women and consequently reduce neonatal infections.

Thirty-five isolates of *Pediococcus* spp. showed antimicrobial activity against *L. monocytogenes*, by production of a bacteriocin, but did not inhibit *S. agalactiae* isolates. *Pediococcus* spp. isolates demonstrated the ability to survive in simulated vaginal fluid at pH 4.2.

Based on the higher bacteriocinogenic activity and survival in simulated vaginal fluid, one isolate of *Pediococcus* spp. was selected and characterized to evaluate its safety before use as vaginal probiotic. *Pediococcus pentosaceus* SB83 did not show the presence of virulence factors such as the production of gelatinase, lipase and DNase, hemolytic activity, nor the presence of virulence genes (i.e. surface adhesin, aggregation protein, cytolysin and extracellular metallo-endopeptidase). No relevant antibiotic

resistance traits were detected. *Pediococcus pentosaceus* SB83 produced biofilms at different pH values (4.2, 5.5 and 6.5) in simulated vaginal fluid, which could also serve as a protective layer against colonization by pathogenic bacteria.

The bacteriocin produced by *P. pentosaceus* SB83, designated as bacteriocin SB83, also showed inhibitory activity against *Enterococcus faecalis* and *Enterococcus faecium*, but did not inhibit vaginal lactic acid bacteria. Bacteriocin SB83 is resistant to several conditions, including conditions in the vaginal tract (pH and components of vaginal fluid).

The bacteriocinogenic activity of *P. pentosaceus* SB83 against *L. monocytogenes* was evaluated in simulated vaginal fluid at pH 6.5, since this is the ideal pH to the *L. monocytogenes* survival and proliferation. There the inhibitory effect of the bacteriocinogenic culture was assessed in suspension, as lyophilized powder and in tablets. Suspensions of *P. pentosaceus* SB83 (10^{10} CFU/mL) reduced the pathogen (10^{5} CFU/mL) after only 2 h of exposure to below the detection limit; the lyophilized bacteria after 24 h of contact and in tablet form, *P. pentosaceus* SB83 lost the antimicrobial activity. The pH of simulated vaginal fluid decreased in all the tested conditions.

Since *P. pentosaceus* SB83 lose its antimicrobial activity in tablet form, it could be therefore used in the form of lyophilized powder, which may be administered intra-vaginally, for instance as a washing solution. This formulation was selected to evaluate the anti-listerial activity during 12 months of storage. During storage at room temperature, lyophilized bacteria totally inhibited the pathogen (below the detection limit) only during one month; after this time, there was a decrease in the cell counts of *P.pentosaceus* SB83 and, consequently, in antimicrobial potential. During storage at 4 ºC, *P. pentosaceus* SB83 showed antimicrobial activity throughout the time of storage investigated. The bacteriocin produced by *P. pentosaceus* SB83 after storage at 4 ºC, remained active at least during 12 months, however a slight decrease in antimicrobial activity occurred between 9 to 12 months. Therefore, the best formulation of *P. pentosaceus* SB83 is as a lyophilized powder stored at 4 ºC.

These *in vitro* results prove a concept for the use of *P. pentosaceus* SB83 as a vaginal probiotic, to prevent vaginal colonization by *L. monocytogenes* in pregnant women.

Resumo

Os compostos antimicrobianos produzidos pelas bactérias do ácido láctico são importantes para a diminuição da colonização vaginal por agentes patogénicos. O pH vaginal é normalmente acídico, variando entre 3,5-4,5; um aumento do pH vaginal (5,0 a 6,5) pode ser associado à colonização por microrganismos patogénicos.

O objetivo principal deste estudo foi selecionar uma cultura com propriedades antimicrobianas com potencial para utilização na prevenção da colonização vaginal por *Listeria monocytogenes* durante a gravidez e, consequentemente, evitar a listeriose neonatal.

Foi investigada a sobrevivência e a capacidade de formação de biofilmes para 20 isolados de *L. monocytogenes* em fluido vaginal simulado a pH vaginal normal (4,2) e a valores de pH elevados (5,5 e 6,5). Este patogénico foi inibido em condições de pH vaginal normal, mas sobreviveu quando este aumentou. Todos os isolados testados formaram biofilme aos diferentes valores de pH.

Streptococcus agalactiae, é um importante agente de colonização maternal e causa de infeção neonatal. Por este motivo, foi também analisado o seu comportamento em condições de simulação de fluido vaginal. Tal como *L. monocytogenes*, os isolados de *S. agalactiae* (n=10) sobreviveram melhor a valores de pH elevados comparativamente ao pH vaginal normal. Todos os isolados foram também produtores de biofilme.

O facto de *L. monocytogenes* e *S. agalactiae* sobreviverem a valores de pH vaginal elevados, sugere que as mulheres grávidas que tenham um aumento do pH vaginal, podem ter um maior risco de infeção neonatal. A produção de biofilmes aumenta a probabilidade de ocorrência destas infeções.

A aplicação vaginal de probióticos apresenta potencial para a prevenção de colonização vaginal por *L. monocytogenes* em mulheres grávidas e, consequentemente, reduzir as infeções neonatais.

Trinta e cinco isolados de *Pediococcus* spp., demonstraram atividade antimicrobiana contra *L. monocytogenes*, pela produção de uma bacteriocina, no entanto, não apresentou atividade contra os isolados de *S. agalactiae*. Estes isolados de *Pediococcus* spp. sobreviveram no fluido vaginal simulado a pH 4,2.

Com base nos resultados da avaliação da atividade bacteriocinogénica e sobrevivência em fluido vaginal simulado, *Pediococcus pentosaceus* SB83 foi selecionado para futura aplicação como probiótico vaginal. *Pediococcus pentosaceus* SB83 não demonstrou

possuir fatores de virulência tais como produção de gelatinase, lipase e DNase, atividade hemolítica, nem possuiu os genes de virulência estudados (adesina de superfície, proteína de agregação, citolisina e metalo-endopeptidase extracelular). Não foram detetadas resistências a antibióticos. *Pediococcus pentosaceus* SB83 demonstrou ser produtor de biofilme em fluido vaginal simulado a diferentes valores de pH (4,2, 5,5 e 6,5), que também pode atuar como camada protetora contra a colonização por bactérias patogénicas.

A bacteriocina produzida por *P. pentosaceus* SB83, designada por bacteriocina SB83, também demonstrou atividade inibitória contra *Enterococcus faecalis* e *Enterococcus faecium*, mas não apresentou efeito inibitório para bactérias do ácido láctico autóctones do trato vaginal. A bacteriocina SB83 demonstrou ser resistente a vários parâmetros, incluindo as condições do trato vaginal (pH e componentes do fluido vaginal).

A atividade bacteriocinogénica de *P. pentosaceus* SB83 contra *L. monocytogenes* foi avaliada em fluido vaginal simulado a pH 6,5, pois este demonstrou ser o pH ideal para a sobrevivência e proliferação de *L. monocytogenes*. Foi testado o efeito inibitório da cultura bacteriocinogénica em suspensão, na forma liofilizada e em comprimidos. A suspensão bacteriana de *P. pentosaceus* SB83 (10^{10} UFC/mL) reduziu o patogénico (10^{5} UFC/mL) em apenas 2 h (abaixo do limite de deteção), a bactéria liofilizada após 24 h de contacto e em comprimidos o *P. pentosaceus* SB83 perdeu a atividade antimicrobiana. O pH do fluido vaginal simulado diminuiu para todas as condições testadas.

Como *P. pentosaceus* SB83 perdeu a atividade antimicrobiana quando na forma de comprimido, poderá ser utilizado na forma liofilizada, a qual pode ser administrada intravaginalmente, por exemplo como uma solução de lavagem. Esta formulação foi a selecionada para avaliar a atividade anti-listerial durante 12 meses de armazenamento.

Durante o armazenamento à temperatura ambiente, a bactéria liofilizada inibiu totalmente o patogénico (abaixo do limite de detecção) apenas durante um mês; após este período de tempo, houve uma diminuição SB83 e, consequentemente, o potencial antimicrobiano. Durante o armazenamento a 4 ºC, *P. pentosaceus* SB83 apresentou atividade antimicrobiana durante todo o tempo de armazenamento investigado. A bacteriocina produzida por *P. pentosaceus* SB83 após o armazenamento a 4 ºC, manteve-se ativa pelo menos durante 12 meses, no entanto, uma ligeira diminuição na atividade antimicrobiana ocorreu entre os 9 e 12 meses. Portanto, a melhor formulação de *P. pentosaceus* SB83 é como liofilizado armazenado a 4 ºC.

Estes resultados *in vitro* provam o conceito para a utilização de *P. pentosaceus* SB83 como probiótico vaginal, para prevenir a colonização vaginal por *L. monocytogenes* em mulheres grávidas.

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Roadmap for the thesis

This thesis consists of six articles, five published in peer-reviewed scientific journals and one is in submission.

The concept of "normal microbiota" as a static and well-defined microbial population is in need of revision, particularly as better information (including that obtained through developed molecular methodologies that are not dependent on culture) is changing the present paradigm. It is recognized that a spectrum of microbial profiles can produce a stable vaginal ecosystem with the ability to maintain vaginal health without succumbing to disease (Farage *et al.*, 2010).

The introductory section covers a general review of the vaginal microbiota and their role in maintaining vaginal health. The incidence of *L. monocytogenes* and its consequences in pregnant women and in fetuses/neonates, is also reported. The importance of the use of lactic acid bacteria as vaginal probiotics to control the colonization by pathogens, is also included.

Section 3, *Survival and biofilm formation of Listeria monocytogenes in simulated vaginal fluid: influence of pH and strain origin*, describes the behavior of *L. monocytogenes* in vaginal fluid *in vitro*, and the influence of the pH on survival of this pathogen. As infection with *L. monocytogenes* in the neonate is very similar to Group B streptococci (*Streptococcus agalactiae*; vertical transmission and clinical manifestations), the behavior of this pathogen in simulated vaginal fluid was also analysed in Section 4, *Survival and biofilm formation by Group B streptococci in simulated vaginal fluid at different pHs*.

Section 5, entitled *Evaluation of characteristics of* Pediococcus spp. *to be used as a vaginal probiotic*, describes the selection of isolates of lactic acid bacteria (*Pediococcus* spp.) with antimicrobial activity against *L. monocytogenes*. In this section, the ability of these isolates to survive and show antimicrobial activity in simulated vaginal fluid, was analysed. The isolate which demonstrated the best results for use as a probiotic, *Pediococcus pentosaceus* SB83, was characterized and was evaluated for its safety, namely, the presence of virulence factors and antibiotic resistance traits.

As *P. pentosaceus* SB83 demonstrated anti-listerial activity by the production of a bacteriocin, in Section 6, *Characterization of a bacteriocin of Pediococcus pentosaceus SB83 and its potential for vaginal application*, bacteriocin SB83 was characterized and evaluated for its stability at different parameters (pH, temperature, detergents) and specifically in vaginal conditions.

Section 7, *Effects of processing and storage on Pediococcus pentosaceus SB83 in vaginal formulations: lyophilized powder and tablets*, describes pharmaceutical formulations for administration of *P. pentosaceus* SB83, namely lyophilized powder and tablets. In this section the stability of bacteria after manufacture and during storage was evaluated, including the viability and the bacteriocinogenic activity against *L. monocytogenes* in simulated vaginal fluid.

The results of this research offer the potential for the use of *P. pentosaceus* SB83 to prevent vaginal colonization by *L. monocytogenes* and furthermore, neonatal listeriosis; these results are discussed in Section 8.

Publications

Borges, S., Silva, J., Teixeira, P. 2013. The role of lactobacilli and probiotics in maintaining vaginal health (submitted for publication).

Borges, S.F., Silva, J.G.L., Teixeira, P.C.M. 2011. Survival and biofilm formation of *Listeria monocytogenes* in simulated vaginal fluid: influence of pH and strain origin. FEMS Immunology and Medical Microbiology 62, 315-320.

Borges, S., Silva, J., Teixeira, P. 2012. Survival and biofilm formation by Group B streptococci in simulated vaginal fluid at different pHs. Antonie van Leeuwenhoeke 101, 677-682.

Borges, S., Barbosa, J., Silva, J., Teixeira, P. 2013. Evaluation of characteristics of *Pediococcus* spp. to be used as a vaginal probiotic. Journal of Applied Microbiology, doi: 10.1111/jam.12232.

Borges, S., Barbosa, J, Silva, J., Teixeira, P. 2013. Characterization of a bacteriocin of *Pediococcus pentosaceus* SB83 and its potential for vaginal application. Anti-Infective Agents 11(2) (in press).

Borges, S., Costa, P., Silva, J., Teixeira, P. 2013. Effects of processing and storage on *Pediococcus pentosaceus* SB83 in vaginal formulations: lyophilized powder and tablets. BioMed Research International, doi: 10.1155/2013/680767.

Keywords

Listeria monocytogenes

Neonatal listeriosis

Pregnancy

Genital tract

Vaginal fluid

Vaginal pH

Vaginal probiotic

Pediococcus spp.

Antimicrobial activity

Bacteriocin

Vaginal administration

Biopharmaceutical product

List of Abbreviations

agg - Aggregation substance gene

ANOVA - Analysis of variance

ATCC - American Type Culture Collection

AU - Arbitrary units

AV - Aerobic vaginitis

BLAST - Basic Local Alignment Search Tool

bp - Base pair(s)

BV - Bacterial vaginosis

CFU - Colony forming unit

cyl - Cytolysin gene

DGGE – Denaturing gradient gel electrophoresis

DNA - Deoxyribonucleic Acid

DNase - Deoxyribonuclease

dNTP - Deoxyribonucleotide triphosphate

DSMZ - German Collection of Microorganisms and Cell Cultures GmbH (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)

ECOFF - Epidemiological cut-off

EDTA – Ethylenediamine Tetraacetic Acid

efaAfm - Cell wall adhesins of *Enterococcus faecium* gene

efaAfs - Cell wall adhesins of *Enterococcus faecalis* gene

EFSA - European Food Safety Authority

EPS - Extracellular polymeric substances

esp - Enterococcal Surface Protein

FDA - Food and Drug Administration

GBS - Group B *Streptococcus*

gel - Gelatinase

GRAS - Generally regarded as safe

HIV - Human immunodeficiency virus

H_2O_2 - Hydrogen peroxide

HPMC - Hydroxypropylmethylcelluloses

LAB - Lactic acid bacteria

MHA - Muller-Hinton agar

MIC - Minimum inhibitory concentration

MRS - de Man, Rogosa and Sharpe

NCCLS - National Committee for Clinical Laboratory Standards

OD - Optical density

PALCAM - PolymyxinAcriflavin-LiCl-Ceftazidime-Aesculin-Mannitol

PCR - Polymerase Chain Reaction

RAPD - Random amplified polymorphic DNA

rRNA - Ribosomal ribonucleic Acid

SDS - Sodium dodecyl sulphate

SDS-PAGE - Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis

SVF - Simulated vaginal fluid

SXT - Trimethoprim/sulphamethoxazole

TAE - Tris, Acetic acid and EDTA

TSA - Tryptone Soya agar

TSB - Tryptone Soya broth

UPGMA - Unweighted pairs group matching algorithm

UTI - Urinary tract infection

WHO - World Health Organization

YE - Yeast extract

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1. Introduction

1.1. Physiology of vagina: the vaginal fluid

The human vagina is often described as slightly S-shaped fibromuscular collapsible tubes between 6 and 10 cm long extending from the cervix of the uterus. The vaginal wall consists of three layers: the epithelial layer, the muscular coat and the *tunica* (Baloglu *et al.*, 2009; Hussain and Ahsan, 2005). The surface of the vagina is composed of various folds, which are often called *rugae*. The *rugae* provide distensibility, support and an increased surface area of the vaginal wall. The vagina has an excellent elasticity because of the presence of smooth elastic fibers in the muscular coat. The loose connective tissue of the *tunica adventia* further increases the elasticity of this organ.

The vaginal fluid is principally constituted for cervical secretion and transudation from the blood vessels with desquamated vaginal cells and leucocytes. Secretions from the endometrium and fallopian tubes also contribute to the vaginal fluid (Hussain and Ahsan, 2005).

Thus, vaginal fluid may include contributions from vaginal transudate, Bartholin's and Skenes's glands, exfoliated epithelial cells, residual urine, and fluids from the upper reproductive tract. The quantity and composition of human vaginal fluid have been studied by many researchers for a diversity of reasons. These include the diagnosis of pathological conditions such as bacterial vaginosis (BV), urinary tract infection (UTI), cancer, premature rupture of membranes during pregnancy, determination of the presence of semen for forensic analysis, determination of the time of ovulation, and the study of organic acids that may act as sexual attractants. Genital tract secretions contain a complex mixture of components, such as various salts, proteins, carbohydrates, low molecular weight organic compounds (Owen and Katz, 1999). Zegels *et al.* (2009)

collected cervical-vaginal fluid during colposcopy and identified 339 proteins, which included antimicrobial peptides such as human β-defensin 2 and cathelicidin. The enzymatic activity in the vagina is comparatively lower than in the gastrointestinal tract but there is still a wide range of enzymes present such as nucleases, lysozymes and esterases (Baloglu *et al.*, 2009).

Previous studies suggest that the daily production of vaginal fluid is around 6 g/day, with approximately 0.5-0.75 g present in the vagina at any one time. The volume of vaginal fluid has been shown to increase substantially during periods of sexual stimulation (Owen and Katz, 1999). The discharge produced by postmenopausal women is reduced by 50% compared to that produced by women of reproductive age. At the time of ovulation, mucus secretion increases and it becomes clear, thin and alkaline (Baloglu *et al.*, 2009).

1.2. The microbiota of the vaginal tract

The species that occupy sites in the human body can change based on intrinsic host factors such as stage of life cycle, hormone levels, immune responses, nutritional status and disease states. The normal microbiota can also be altered by external factors such as environmental exposures, microbial interspecies competition or commensalism, and hygiene behaviors (Bolton *et al.*, 2008).

The first extensive study of human vaginal microbiota was published by Döderlein in 1892. Döderlein considered the vaginal microbiota to be homogenous, consisting only of gram-positive bacilli (Döderlein, 1892). These Döderlein's bacilli are now known to be members of the genus *Lactobacillus* spp.. This concept has been modified by researchers who have found the microbiota of asymptomatic women to consist usually of a diversity of anaerobic and aerobic microorganisms. The understanding of

compositions and structure of the vaginal microbiome has significantly broadened as a result of using cultivation-independent methods based on the analysis of 16S ribosomal RNA (rRNA) gene sequences (Ma *et al*., 2012).

Lactobacilli are the most prevalent and often numerically dominant microorganisms, at 10^7 to 10^8 CFU/mL of vaginal fluid in healthy premenopausal women (Boris and Barbés, 2000; Farage *et al.*, 2010).

Among the lactobacilli species found in the vaginal microbiota, *Lact. iners, Lact. crispatus, Lact. gasseri, Lact. jensenii,* followed by *Lact. acidophilus, Lact. fermentum, Lact. plantarum, Lact. brevis, Lact. casei, Lact. vaginalis, Lact. delbrueckii, Lact. salivarius, Lact. reuteri, and Lact. rhamnosus* are the most frequently isolated from healthy women (Cribby *et al.*, 2008). Individual differences in *Lactobacillus* spp. composition of the vaginal tract between women of different geographic locations, races and ethnicities have been noted across multiple studies (Table 1.1.). In a study by Ravel *et al*. (2011), the vaginal bacterial communities of asymptomatic North American women who represented four ethnic groups (white, black, Hispanic and Asian) were characterized by pyrosequencing of barcoded 16S rRNA genes and the vaginal microbiota varied among the four ethnic groups.

In addition to multiple species of *Lactobacillus* spp., other lactic acid bacteria (LAB) genera have been found in the vaginal tract, such as *Pediococcus* spp., *Weisella* spp., *Streptococcus* spp. and *Leuconostoc* spp. (Jin *et al.*, 2007).

Table 1.1. Prevalence of *Lactobacillus* spp. in vaginal tract of different women (expressed in percentage values).

***Lactobacillus* spp.**	**Antonio *et al.* (1999) (Seatle, n=302)**	**Anukam *et al.* (2006) (Nigeria, n=24)**	**Aslim and Kilic (2006) (Turkey, n=10)[a]**	**Tamrakar *et al.* (2007) (Japan, n=98)**	**Vitali *et al.* (2007) (Belgium, n=26)**	**Brolazo *et al.* (2009) (Brazil, n=135)[b]**
Lact. crispatus	32	3.0	14	61.2		30.1
Lact. jensenii	23		3	29.6		26.5
Lact. gasseri	5	7.3	21	33.7	present	22.9
Lact. ruminis	0.3					
Lact. reuteri	0.3					2.4
Lact. fermentum	0.3	1.3				2.4
Lact. oris	0.3		2			
Lact. vaginalis	0.3	2.7	16		present	8.4
Lact. iners		64.4		39.8	present	
Lact. plantarum		6.0	5			
Lact. suntoryeus		6.0				
Lact. rhamnosus		2.7				2.4
Lact. helveticus		1.3				
Lact. johnsonii		1.3				
Lact. salivarius		1.3	3			1.2
Lact. acidophilus			16		present	
Lact. delbrueckii			14			2.4
Lact. cellobiosus			3			
Lact. curvatus			2			
Lact. brevis			2			
Lact. mucosae						1.2
No homology	4					
No lactobacilli	29					
***Lactobacillus* spp.**	15	2.7				
Methodology	whole-chromosomal DNA probes to 20 *Lactobacillus* strains	processed by denaturing gradient gel electrophoresis (DGGE) and identified by DNA sequencing	biochemical tests	PCR with primers for 16S ribosomal DNA	DGGE and real-time PCR analysis	multiplex PCR

a) Percentage relative to the number of isolates (58 isolates of *Lactobacillus* spp.)

b) Percentage relative to the number of isolates (83 isolates of *Lactobacillus* spp.)

The vaginal vault is colonized within 24 hours of a female child's birth and remains colonized until death (Farage *et al.*, 2010). Lactobacilli become the predominant inhabitants of the vagina at the time of puberty, presumably because of the effect of estrogens on the glycogen content of vaginal epithelial cells (Schwebke, 2001). Menopause is marked by a dramatic reduction in estrogen production, resulting in drying and atrophy of the vaginal epithelium. When estrogen levels drop, glycogen content in the vaginal epithelium drops as well, leading to depletion of lactobacilli. Diminishing numbers of lactobacilli result in a subsequent rise in vaginal pH, since glucose is not converted to lactic acid. High pH values promote growth of pathogenic bacteria, particularly colonization by enteric bacteria (Farage *et al.*, 2010). Burton and Reid (2002) performed a study with 20 postmenopausal women (range 44-72 years) and 70% had either intermediate-grade bacterial colonization or BV.

Therefore, the vaginal microbial ecosystem suffers significant structural changes at various stages in a woman's life that are directly associated to the level of estrogen in the body (Hickey *et al.*, 2012).

The complex interactions of the various members of the vaginal microbiota are not well understood; however, it is recognized that the composition of the microbiota can vary from day to day, even in women without indication of infections. Studies using sequential vaginal cultures or vaginal smears have shown that in some women, there are significant, transient changes in the microbiota. The most unstable time appears to be around the time of menses, but the exact factors responsible for these changes are unknown (Schwebke, 2001).

The microorganisms that inhabit the vaginal environment, play a major role in preventing illnesses of the host, including BV, UTI, yeast vaginitis, and sexually

transmitted diseases including human immunodeficiency virus (HIV) (Reid and Bocking, 2003).

There seems to be an association between absence (or low concentrations) of vaginal lactobacilli and the development of BV. Compared with that of normal women, the vaginal microbiota of women with BV consists more commonly, and in higher numbers, of *Gardnerella vaginalis*, *Mycoplasma hominis*, *Prevotella* spp., *Peptostreptococcus* spp., *Mobiluncus* spp., *Bacteroides* spp., *Atopium vaginae* and *Megasphera* spp. (Cribby *et al.*, 2008, Falagas *et al.*, 2007). Bacterial vaginosis is characterized by the presence of three of five criteria (Amsel criteria): release of an amine fishy odor, release of amine odor after addition of 10% potassium hydroxide, vaginal pH values greater than 4.5, clue cells in the vaginal fluid, and milky homogenous vaginal discharge (Reid and Bocking, 2003). Nugent *et al.* (1991) suggested that BV should be diagnosed by vaginal smears examined following Gram's stain. The examination consists of scoring the cell population as to being normal (0 to 3) and dominated by *Lactobacillus* spp., intermediate (4 to 6) with colonization by small gram-negative or gram-variable rods (*Bacteroides* spp. or *G. vaginalis*) and curved gram-variable rods (*Mobiluncus* spp.), and BV (7 to 10) with domination by pathogens and absence of lactobacilli.

Bacterial vaginosis is associated with high pH, a decrease in antimicrobial activity of the vaginal fluid compared to healthy women, and local impairment of the multiple innate immune pathways (Dover *et al.*, 2008). A typical feature of BV is the absence of inflammation. In BV, there is only a slight increase in interleukin I and a low production of interleukin 8, preventing the attraction of inflammatory cells such as macrophages and neutrophils (Donati *et al.*, 2010).

A second major abnormality of the vaginal microbiota, denominated as aerobic vaginitis (AV), has been described. In this disorder, the normally present *Lactobacillus* spp. are replaced with aerobic organisms, predominantly enteric commensals or pathogens. Such a community was most often classified as "intermediate". Group B streptococci (GBS), *Escherichia coli*, and *Staphylococcus aureus* are the organisms most frequently associated with AV (Rampersaud *et al.*, 2012).

Donders *et al.* (2002) proposed the following microscopic features to diagnose this disorder: (1) a paucity of lactobacilli; (2) an increased number of leukocytes; (3) the presence of parabasal cells (a sign of epithelial inflammation); and (4) the presence of cocci or coliform bacteria.

Despite the proximity of the vagina to the anus, the variety of microorganisms present in the vagina is much lower than in the gut. The reduced receptivity of the vagina, different nutrient availability and competition with indigenous organisms can be the reasons for this lower diversity in vaginal tract. Some microbes found in the gut, can also be found in the vagina, demonstrating the proper receptors, nutrients, and oxygen tension are present for these organisms to grow (Cribby *et al.*, 2008).

Accessible data about types and numbers of bacteria present in the vagina have not correlated well with clinical symptoms, an observation which may well be the result of the incomplete analysis of the vaginal ecosystem available through traditional methods.

Development of molecular techniques that can identify (without the need for laborious microscopy or prior culture) the organisms comprising the vaginal microbial community, are proving useful in defining organisms that have defied identification through traditional cultural methods (Farage *et al.*, 2010).

1.3. Vaginal microbiota and pregnancy

Numerous types of microorganisms which can be found in association with preterm labor and delivery, such as *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Trichomonas vaginalis*, *Haemophilus influenza* only occasionally acts like a pathogen, being associated with preterm labor. Group B streptococci can have devastating effects on the preterm or low birth weight infant. A number of genital microorganisms such as *E. coli*, *L. monocytogenes* and viridans streptococci may be involved in chorioamnionitis. Bacterial vaginosis is a polymicrobial condition increasingly associated with adverse perinatal sequelae (Donati *et al.*, 2010).

The importance of LAB in life is perhaps best seen in relation to the health of women and babies (Reid, 2008). Lactobacilli are able to reduce the incidence of ascending infections of the uterus and the subsequent production of proinflammatory molecules (Wilks *et al.*, 2004).

Once pregnant, the loss of lactobacilli from the vagina and subsequent development of BV is associated with serious complications include preterm delivery of low birth weight infants, spontaneous abortion, premature rupture of membranes, preterm birth, amniotic fluid infections, postpartum endometritis and endometritis following Caesarian section (Hickey *et al.*, 2012). Toxins from BV-associated microorganisms (such as lipopolysaccharides) may cross the placenta and cause brain injuries in fetuses. The toxins may cause permanent neurological brain damage such as cerebral palsy, a risk of developing Parkinson's disease and schizophrenia (Dover *et al.*, 2008).

In terms of pregnancy, BV has been associated with a 40% increased risk of preterm labor and it occurs in approximately 16% of the untreated female population. Yet, the treatment of this common vaginal infection with antibiotics may or may not decrease the incidence of premature labor. Standard antibiotic therapy for BV with metronidazole

is quite ineffective in that more than 30% of women have yeast vaginitis after therapy and more than 50% get a recurrent BV infection within 3 to 6 months. The failure of antibiotic therapy for BV to prevent preterm delivery may reflect organisms already having ascended the uterus or antibiotics being unable to eradicate BV biofilms and negate their sialidase activity (Reid and Bocking, 2003).

Moreover, the healthy vaginal microbiota is disturbed by antibiotics and the risk of developing antimicrobial drug resistance increases dramatically with overall increased use of antimicrobial preparations (feminine hygiene and treatment). *In vitro* studies have shown that clindamycin and metronidazole inhibit *Lactobacillus* spp. at concentrations lower than doses topically applied for treatment. Therefore, there is an interest in developing alternative treatments against BV. Local treatment of BV with no systemic effects would be safer for pregnant women (Dover *et al.*, 2008).

Prospective analyses have linked AV with several pregnancy-related complications including late miscarriage, chorioamnionitis, and preterm birth. The precise role of AV during pregnancy, the utility of screening, and potential therapies require further study (Rampersaud *et al.*, 2012).

The placenta, fetal membranes, and cervical mucus have a collective function to defend the fetus from pathogenic microorganisms. Several adverse obstetric outcomes including miscarriage, chorioamnionitis, premature rupture of membranes and preterm birth have been associated with presence of bacteria in the intrauterine cavity. These consequences are supposed to be the direct result of the maternal and sometimes fetal inflammatory response to bacterial pathogens. It is difficult to establish whether bacteria may exist in these tissues without inducing a deleterious inflammatory response (i.e. colonization without infection). Because rupture of the fetal membranes and uterine contractions are independently associated with microbial invasion of the intrauterine

cavity, only studies using specimens obtained from women with intact membranes, prior to the onset of labor, can effectively address this question. Therefore, the majority of investigations are made in women delivering preterm secondary to maternal indications, such as pre-eclampsia, or in those presenting for elective cesarean section at term gestation. Variability in the specimen collected (placental tissues, fetal membranes or amniotic fluid) and methodologies employed for bacterial detection (culture-dependent and molecular-based techniques) contribute to the difficulty in interpreting reported data (Rampersaud *et al.*, 2012).

1.4. *Listeria monocytogenes* and pregnancy

There are multiple species of *Listeria* including *L. monocytogenes, L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. grayi* and *L. murrayi*. Despite the range of species, only *L. monocytogenes* can cause disease in humans. *Listeria monocytogenes* is a Gram-positive rod that is motile via flagella, catalase positive, oxidase-negative and a facultative anaerobe. It is distinguished from other species of *Listeria.* in that it forms a narrow zone of β-hemolysis. In some cases, *L. monocytogenes* can be Gram-variable and resemble cocci, diplococci, or diphtheroids, which may lead to a diagnosis of streptococcal or enterococcal infection (Delgado, 2008; DiMaio, 2000).

Infection with *L. monocytogenes* can be acquired from food, animals through direct contact, via vertical transmission from mother to fetus/neonate through the placenta or birth canal, and via nosocomial cross-infection (Delgado, 2008). The oral bacterial load required to cause infection has been estimated to be greater than 10^4. The vagina, cervix, and pharynx are other sites for potential carriage of the microorganism (DiMaio, 2000).

Listeria monocytogenes can cause systemic disease among immunocompromised persons, the elderly, pregnant women and neonates. In United States, the annual incidence of listeriosis is 0.7 per 100,000 in the general population and 12 per 100,000 in pregnant women (a 17-fold increase). Listeriosis has an overall mortality rate of 20% to 23% (Delgado, 2008), but may be as high as 50% in the neonatal population. If a mother becomes infected with *L. monocytogenes* the fetus is affected in more than 90% of cases (DiMaio, 2000). In Portugal, listeriosis is not a notifiable infection and available data are scarce. In a study, data from 23 hospitals and a National Institute of Health delegation in Portugal, were recorded; 35 cases of listeriosis were identified for 1994-2003 and the mortality rate was greater than 17%. In 2003, the incidence of listeriosis in Portugal was at least 1.4 cases per million inhabitants (Almeida *et al.*, 2006).

Generally, maternal illness is mild and sometimes even asymptomatic (Janakiraman, 2008). Common signs and symptoms include fever, muscle aches, nausea and vomiting, and diarrhoea (Delgado, 2008). However, listeriosis during pregnancy has serious consequences such as spontaneous abortion, stillbirth, premature delivery and neonatal infection. Neonatal disease can be divided into early or late onset listeriosis. In early onset, symptoms appear in 1.5 days and in late onset appear after several days to weeks. Early-onset disease is probably acquired *in utero*, due to the bacteremic phase of their mothers or by ascending infection. Early-onset disease often presents in the preterm infant with sepsis, respiratory distress, purulent conjunctivitis and skin lesions (Delgado, 2008; DiMaio, 2000). The highest concentrations of *L. monocytogenes* are found in the lung and gut, suggesting that infection can be acquired via inhalation and ingestion of infected amniotic fluid as well as via the hematogenous route (Posfay-Barbe and Wald, 2009).

Late onset disease is more likely in term infants who acquired *L. monocytogenes* from the vaginal tract of asymptomatic mothers, during passage through the birth canal (Delgado, 2008; DiMaio, 2000). The clinical manifestation in this group is more likely to be meningitis than sepsis, but it can be subtle, with fever, irritability, anorexia, diarrhea, and lethargy (Posfay-Barbe and Wald, 2009). Worldwide, *L. monocytogenes* is one of the three major causes of meningitis in neonates (Lecuit, 2007). Listeriosis in the neonate is very similar to GBS infection in both clinical manifestations and treatment (Delgado, 2008).

After birth, microscopic abscesses on the placenta or amnionitis in the pathology report can be indicative of *L. monocytogenes*. As some women have no symptoms or nonspecific, it is possible to miss cases of *L. monocytogenes* infection (Delgado, 2008). Physicians seldom take cultures from aborted tissue or fetus, which may also result in missing the diagnosis of perinatal infection, and many cases of neonatal listeriosis are not published. Thus, the incidence of perinatal infection may be underestimated (Chen *et al.*, 2007).

The illness is usually self-limiting and mild in the mother, therefore treatment is aimed at preventing obstetric complications and improving neonatal outcomes. Prompt treatment during pregnancy can significantly decrease the rate of fetal infection and morbidity and mortality in the neonate. The antibiotic therapy of choice is a high dose of intravenous ampicillin, for neonatal listeriosis, treatment should be extended to 3-4 weeks. Trimethoprim-sulfamethoxazole is recommended in the pregnant patient with penicillin allergy. Cephalosporins and clindamycin, which usually work against Gram-positive pathogens, are not effective against *L. monocytogenes.* Aminoglycoside is unlikely to be effective and the recommendations may differ. Although *L. monocytogenes* is reported to be susceptible to meropenem *in vitro*, treatment failure of

meropenem in pediatric patients has been documented (Chen *et al.*, 2007; Delgado, 2008; DiMaio, 2000). Prenatal vitamins or iron supplementation should be temporarily discontinued because iron appears to enhance the virulence of the microorganism (DiMaio, 2000).

Due to the nonspecific symptoms and the serious consequences in pregnancy, prevention of infections by *L. monocytogenes* should be the primary focus.

1.5. Role of *Lactobacillus* spp. in maintaining vaginal health

1.5.1. Antimicrobial activity

The role that the vaginal ecosystem plays in the maintenance of vaginal health is important in a functional equilibrium. This healthy equilibrium acts to supply a barrier to new colonization by pathogenic organisms and overgrowth of organisms that are otherwise commensal. The mechanisms by which lactobacilli stabilize the vaginal microbiota are the production of antimicrobial compounds (hydrogen peroxide, lactic acid, bacteriocin-like substances) and the capability to adhere and compete for adhesion sites in the vagina.

1.5.1.1. pH

The role that vaginal LAB play in inhibiting pathogens could be linked to their ability to acidify the vaginal tract (Charlier *et al.*, 2009). The pH may become more alkaline as the microbiota shifts toward BV and may be affected by other factors, such as menses, douching, and semen (Schwebke, 2001). The vaginal pH varies from 6.6 (+/- 0.3) to 4.2 (+/- 0.2) between day 2 and day 14 of the menstrual cycle. The production of lactic acid by LAB can contribute to this acidification, nevertheless, this low pH is also maintained

by the secretion of organic acids by the vaginal epithelial cells themselves (Charlier *et al.*, 2009).

Acid production has long been known to be detrimental to some microorganisms, not only killing viruses such as HIV, rotavirus and even influenza virus, but also displacing some pathogens from surfaces. Some microorganisms in the vagina, such as yeast and enterococci, can tolerate acids and resist hydrogen peroxide action. This may be due to cell wall structures and biofilm formation. The end result is that very few probiotic strains are effective against *Candida* and enterococci in the vagina (Reid *et al.*, 2006).

There are evidences to suggest that the presence of *Lactobacillus* spp., the production of lactic acid, and the resulting low pH are important for preventing the colonization and proliferation of non-indigenous organisms in the vagina. However, these observations have been over-interpreted and have led to the statement that *Lactobacillus* spp. must be present for maintained vaginal health. When women have a lack of *Lactobacillus* spp. in the vaginal community, this situation is considered abnormal. This fallacy is the premise of the Nugent criteria used for the diagnosis of BV in which the degree of ‘‘healthiness’’ is assessed by the abundance of *Lactobacillus* morphotypes, ignoring the possibility that their ecological function could be surpassed by bacteria with other morphotypes. It might be more logical to assume that an ecological function of vaginal communities, specifically the production of lactic acid, might be accomplished by a variety of taxa capable of homolactic and heterolactic fermentation of substrates (Hickey *et al.*, 2012).

1.5.1.2. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is an oxidizing agent, toxic to catalase-negative bacteria such as most anaerobic microorganisms. It has been suggested that H_2O_2 may also help

prevent invasions since some vaginal species of *Lactobacillus* produce H_2O_2. Antonio *et al*. (1999) found that H_2O_2 was produced by 95% of *Lact. crispatus* and 94% of *Lact. jensenii* vaginal isolates. Aroutcheva *et al.* (2001) reported that approximately 80% of the strains of vaginal origin produced H_2O_2.

There were striking differences between the amounts of H_2O_2 production, Aslim and Kilic (2006) reported a range of 1.01 to 15.50 µg/mL H_2O_2.

Some studies *in vitro* suggest that women with H_2O_2-producing lactobacilli are less likely to be infected with HIV-1, herpes simplex virus type 2 (Conti *et al.*, 2009) and pathogens associated with BV (Matu *et al.*, 2010). However, H_2O_2-producing strains, deemed by some researchers to be critical for vaginal defense, can activate HIV-1 and increase production of intact virions (Klebanoff *et al.*, 1999).

The H_20_2-producing microorganisms that are present in the vagina of healthy women have been suggested as the bacterial group that is responsible for the maintenance of the ecologic balance, mainly in pregnant women. Wasiela *et al*. (2008) demonstrated that the presence of H_20_2 producing lactobacilli (*Lact. acidophilus*, *Lact. fermentum*, *Lact. minutus*, *Lact. jenseni* and *Lact. fermentum*) seems to protect against the development of BV during pregnancy. Pregnant women with BV lack lactobacilli, especially H_20_2-producing strains. Kim *et al.* (2006) observed that the distribution of H_20_2-producing lactobacilli in vaginal microbiota, as defense factors for infection, may have an important role in the pathophysiology of preterm labor.

Some studies have shown this is not likely to be the case *in vivo*, since dissolved oxygen levels in the vagina are exceptionally low and may limit the amount of H_2O_2 produced by lactobacilli to sub-inhibitory levels (Hickey *et al.*, 2012; Muench *et al.*, 2009). Furthermore, O'Hanlon *et al*. (2010) measured the H_20_2 concentration from women with H_20_2-producing lactobacilli, using fluorescence and *in vitro* bacterial-exposure

experiments, and this activity was blocked with cervicovaginal fluid and semen. According to this study, it is improbable that H_2O_2-production by vaginal lactobacilli is a significant mechanism of protection *in vivo*.

Therefore, clinical studies concerning the role of H_20_2-producing lactobacilli are controversial.

1.5.1.3.Bacteriocins

Bacteriocins (antimicrobial peptides or proteins) are produced by almost all genera of LAB, and can be divided into different classes based on their biochemical properties. Class I bacteriocins are the lantibiotics, which are small and post-translationally modified to contain amino acids such as lanthionine and β-methyllanthionine, and several dehydrated amino acids. Class II includes unmodified heat-stable bacteriocins containing peptides with molecular masses of <10 kDa. Class II are subdivided into three subgroups, namely, class IIa (pediocin-like bacteriocins), class IIb (two-peptide bacteriocins), and IIc (other one-peptide bacteriocins). The class III peptides are thermo-sensitive proteins with molecular masses of >30 kDa (Drider *et al.*, 2006; Riley and Wertz, 2002). Another group, known as class IV, is often included in classification. Bacteriocins of class IV are complex molecules with lipid and carbohydrate moieties (Papagianni and Anastasiadou, 2009).

Bacteriocins have a variety of killing mechanisms, including cytoplasmic membrane pore formation, interference with cellular enzymatic reactions (such as cell wall synthesis) and nuclease activity (Gillor *et al.*, 2005).

Nisin (a lantibiotic produced by certain strains of *Lactococcus lactis*) is the only bacteriocin that has been approved by the World Health Organization (WHO) for use as

a food preservative. However, nisin may not be a good choice for vaginal application as it is strongly bactericidal for the healthy vaginal *Lactobacillus* spp. (Dover *et al.*, 2008). Lactic acid bacteria strains have also been screened for bacteriocins production, in order to develop vaginal probiotics (Charlier *et al.*, 2009).

A few bacteriocins from vaginal isolates of *Lactobacillus* spp. have been identified. Ocaña *et al.* (1999) found a vaginal *Lact. salivarius* subsp. *salivarius* CRL 1328 that produced a bacteriocin with activity against *Ent. faecalis*, *Ent. faecium* and *N. gonorrhoeae*.

Aroutcheva *et al.* (2001) studied 22 vaginal isolates of *Lactobacillus* spp., of which approximately 73% exhibited bacteriocin activity against *G. vaginalis*. In that study, it was observed that not all strains of *G. vaginalis* responded identically to the bacteriocinogenic activity of lactobacilli. The authors believe that this difference in the ability of *G. vaginalis* to resist the action of bacteriocin may be responsible for the more resistant cases of BV.

Pascual *et al.* (2008a) studied a *Lact. fermentum* strain L23 that produced a small bacteriocin which displayed a wide inhibitory spectrum including both Gram-negative and Gram-positive pathogenic strains and two species of *Candida* spp..

Vera Pingitore *et al.* (2009) described a bacteriocin produced by vaginal *Lact. salivarius* CRL 1328 that exhibited activity against *Ent. faecalis* by dissipating membrane potential and trans-membrane proton gradient (both components of proton motive force).

However, some studies reported that the bacteriogenic activity of vaginal isolates, that inhibited the healthy vaginal microbiota, lead to the disruption of the ecology of the healthy vagina and paved the way for the establishment of the abnormal microbiota associated with BV. Dezwaan *et al.* (2007), characterized a bacteriocin produced by a

vaginal isolate of *Ent. faecium* strain 62-6; this bacteriocin had a wide spectrum of growth inhibition against Gram-positive bacteria (corynebacteria, streptococci, enterococci) of vaginal origin including lactobacilli. In a study performed by Karaoğlu *et al.* (2002), 100 *Lactobacillus* spp. strains were isolated from vaginal samples of 75 reproductive women and 6 had bacteriocin activity against some common pathogenic bacteria colonized in the human intestine and vagina (*G. vaginalis* and *Pseudomonas aeruginosa*) but also inhibited vaginal lactobacilli. Therefore, the natural inhibition of vaginal lactobacilli by bacteriocins may be important in understanding the initiation of vaginal infections or BV associated with an unexplained decrease of vaginal lactobacilli.

1.5.2. Adherence

The capacity that lactobacilli have to adhere and compete for adhesion sites in the vaginal epithelium, can also be involved in the inhibition of colonization by a pathogen. The renewal of the superficial epithelium of the vagina can affect the equilibrium of the vaginal microbiota (Charlier *et al.*, 2009). Factors such as hormonal changes (particularly estrogen), vaginal pH, and glycogen content can all affect the ability of lactobacilli to adhere to epithelial cells and colonize the vagina. The menstrual cycle can also cause changes in the vaginal microbiota, with high concentrations of estrogen increasing adherence of lactobacilli to vaginal epithelial cells (Cribby *et al.*, 2008).

In the healthy urogenital tract, it is believed that indigenous lactobacilli exclude the colonization of pathogenic bacteria by occupying or masking (by steric hindrance) their potential binding sites in the mucosa. However, in a depleted lactobacilli environment such as an infected urogenital tract, it should be supposed that exogenous probiotic lactobacilli have the capacity to compete for the same receptors and displace previously

attached pathogens. The blockage of urogenital pathogens adherence by lactobacilli may be by exclusion, competition for receptor sites and displacement of adhered pathogens. Several works have shown the ability of *Lactobacillus* spp. to adhere to epithelial vaginal cells in order to form a biological barrier against colonization by pathogenic bacteria (Coudeyras *et al.*, 2008; Zárate and Nader-Macias, 2006). It has been considered that multiple components of the bacterial cell surface participate in this process. Boris *et al.* (1998) reported that the factors responsible for adherence to epithelial vaginal cells seemed to be glycoproteins and carbohydrates.

Self-aggregation may substantially increase the colonization potential of lactobacilli in environments with short residence times. Therefore, both self-aggregation and adhesion may favor the colonization of the vaginal epithelium through the formation of a bacterial film (Boris *et al.*, 1998).

1.6. Potential of lactic acid bacteria for use as vaginal probiotics

The term probiotic derives from the Greek/Latin word “pro” and the Greek word “bios”, meaning for life. The concept of probiotic was possibly firstly introduced by the Russian Nobel laureate Elie Metchnikoff in 1907 (“The Prolongation of Life: Optimistic Studies”) where he proposed the idea that ingesting microbes could have beneficial effects for human beings, especially to treat digestive diseases. The term “probiotic” was first used in 1965, by Lilly and Stillwell, to describe substances secreted by one organism which stimulates the growth of another. The World Health Organization and the Food and Agriculture Organization of the United Nations have defined probiotics as “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host” (Iannitti and Palmieri, 2010).

A remarkable interest from women was shown on the potential use of LAB for maintaining normal urogenital health (Anukam, 2007). A probiotic may act indirectly through treating and preventing recurrent BV or directly by secreting substances (e.g. hydrogen peroxide, bacteriocins, lactic acid) that block sexually transmitted infection (Bolton *et al.*, 2008) (Figure 1.1).

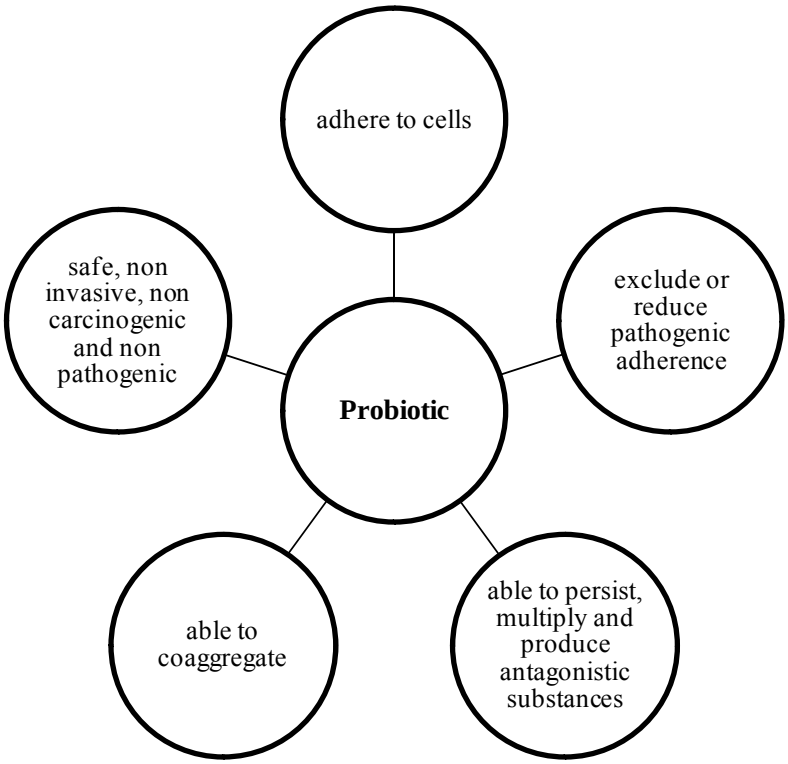

Figure 1.1. Requirements in the choice of a probiotic (Iannitti and Palmieri, 2010).

As antimicrobial treatment of urogenital infections is not always effective, and problems remain due to bacterial and yeast resistance, recurrent infections, as well as side effects, alternative drugs are of interest to patients and their caregivers. It is assumed that recurrences are due to antimicrobials failing to eradicate the pathogens, maybe because of biofilm resistance, or that the virulent organisms come back from their source (the person's gut or a sex partner) and attack a host whose defenses are suboptimal (Cribby *et al.*, 2008).

Several clinical trials have been performed to investigate whether specific strains of lactobacilli, administered either orally (Reid *et al.*, 2004) or intra-vaginally (Ehrström *et al.*, 2010; Marcone *et al.*, 2010), are able to colonise the vaginas of women with symptomatic or asymptomatic BV, to reduce the colonization of pathogens, and to improve symptoms and/or signs of BV when they are present (Falagas *et al.*, 2007). Probiotics can be administered via direct instillation into the vagina or orally because lactobacilli can ascend passively from the rectum to the vagina, which can be a significant breakthrough in being able to deliver probiotics in foods and dietary supplements (Reid, 2008). The time for this intervention to affect the vaginal tract is evidently longer than direct vaginal instillation, and will depend on viability of the strains as they pass through the stomach and gut. In addition, the load of lactobacilli that can be delivered this way is obviously lower than via vaginal administration. However, an advantage of the oral approach may be the ability of the lactobacilli to reduce the transfer of yeast and pathogenic bacteria from the rectum to the vagina, which could potentially lower the risk of infection (Cribby *et al.*, 2008).

Vaginal dosage forms available around the world include creams, gels, tablets, capsules, pessaries, foams, ointments, films, tampons, rings, and douches (Table 1.2). While the majority of vaginal drugs so far have been in the form of gels, there is a growing interest in alternative dosage forms such as rings, tablets, and films. The majority of issues with the product development and scale up are similar to other pharmaceutical products, although there are some unique challenges because of site of delivery, prophylactic nature of product application, and diversity in sex and hygiene practices across the developing world (Garg *et al.*, 2010).

Table 1.2. Several studies with different dosage forms for vaginal probiotics.

Vaginal drug delivery systems	Microrganism	Quantity	Other compounds	Reference
Tablet	*Lact. brevis* or *Lact. salivarius* or *Lact. crispatus* or *Lact. gasseri*	The initial quantity in each tablet is not described. But the maximum found after one month of storage at 4 °C is 10^9 cells/g	**Fast-release layer:** Lactose, maize starch, adipic acid, sodium bicarbonate, ascorbic acid, stearic acid, magnesium stearate and colloidal silicon dioxide **Slow-release layer:** Ascorbic acid, mannitol, retarding polymer, talc, magnesium stearate and colloidal silicon dioxide	Maggi *et al.* (2000)
Capsule	*Lact. fermentum* RC-14 and *Lact. rhamnosus* GR-1	$1x10^9$ total CFU per capsule		Burton *et al.* (2003)
Tampons	*Lact. gasseri* *Lact. casei* var *rhamnosus* *Lact. fermentum*	10^8 living bacteria per tampon		Eriksson *et al.* (2005)
Suppository	*Lact. crispatus* CTV-05	$5x10^8$ CFU for suppository	Preservation matrix and maltodextran	Czaja *et al.* (2007)
Douche vaginal	*Lact. acidophilus*	$1x10^9$ CFU/mL (final bacterial concentration after suspension in 100 mL of water)	10% hydroxypropyl guar gum and 9.5% NaCl	Drago *et al.* (2007)
Tablet	*Lactobacillus* spp.	Not described	Citric acid, sodium bicarbonate and lactose	Kale *et al.* (2008)
Gelatin capsules	*Lact. gasseri* *Lact. rhamnosus*	10^8-10^9 CFU/capsule	Lactitol monohydrate, gelatine, cornstarch, xanthan gum, glucose anhydrous, titanium dioxide and magnesium stearate	Larsson *et al.* (2008)
Suppositories	*Lact. paracasei* HL32	10^8 CFU for suppository	Witepsol H-15 or mixed polyethylene glycols	Kaewnopparat and Kaewnopparat (2009)
Capsule	*Lact. gasseri* LN40, *Lact. fermentum* LN99, *Lact. casei subsp. rhamnosus* LN113 and *Pediococcus acidilactici* LN23	Between 10^8 and 10^{10} viable cells per capsule	Maltodextrin and magnesium stearate	Ehrström *et al.* (2010)

1.6.1. Clinical trials

Some randomized controlled trials reported enhanced cure rates or a reduced recurrence of BV among premenopausal women treated with a vaginal probiotic.

Marcone *et al.* (2010) investigated the advantages of long-term vaginal administration of *Lact. rhamnosus* after oral treatment with metronidazole to prevent the recurrence of BV. A total of 49 women were diagnosed with BV, some of them were treated either with oral metronidazole for 7 days followed by a once-weekly vaginal application of 40 mg of *Lact. rhamnosus* for 6 months. During the first 6 month of follow-up, 96% of patients had a balanced vaginal ecosystem. The vaginal probiotic allowed stabilization of the vaginal ecosystem and reduced the recurrence of BV.

Drago *et al.* (2007) treated 40 women with BV for 6 days with a douche containing *Lact. acidophilus*. The Nugent score decreased significantly from BV, and remained low during the follow-up period for almost all of the patients, indicating BV in 52.5% and in 7.5% of the patients before treatment and at follow-up, respectively. After treatment, significant decreases in vaginal pH were observed, to less than pH 4.5 in 34/40 women, and the odor test became negative in all of the patients.

Ehrström *et al.* (2010) reported a randomized double-blind placebo controlled study to assess the vaginal colonization of LAB and clinical outcomes. Vaginal capsules containing *Lact. gasseri* LN40, *Lact. fermentum* LN99, *Lact. casei* subsp. *rhamnosus* LN113 and *P. acidilactici* LN23 (10^8 and 10^9 viable cells for capsule) were administered for five days to 95 women after conventional treatment of BV and/or vulvovaginal candidiasis. Probiotic strains were present 2-3 days after administration in 89% of the women. After one menstruation 53% were colonized by at least one LN strain. Nine percent were still colonized six months after administration. The probiotic

supplementation resulted in less malodorous discharge, and a trend towards higher clinical cure rate, compared with the placebo group.

However, some trials found no beneficial effects of vaginal probiotic in the treatment of BV. Eriksson *et al.* (2005) performed a double-blind placebo-controlled study (187 patients) of adjuvant lactobacilli treatment after an open treatment with vaginal clindamycin ovules. There was no improvement in the cure rate after treatment with lactobacilli-containing tampons compared to placebo tampons; the cure rates as defined by Amsel's criteria were 56% and 62%, respectively, and 55% and 63%, as defined by Nugent's criteria.

In a study by Larsson *et al*. (2008), 100 women with BV diagnosed by Amsel criteria, after informed consent were offered vaginal clindamycin therapy followed by vaginal gelatine capsules containing either 10^9 freeze-dried lactobacilli or identical placebo capsules for 10 days during three menstrual cycles in a double-blind, randomized, placebo-controlled trial. The study showed that supplementary treatment combining two different strains of probiotic lactobacilli did not improve the efficacy of BV therapy during the first month of treatment, but for women initially cured, adjunct treatment of lactobacilli during three menstrual cycles lengthened the time to relapse significantly in that more women remained BV-free at the end of the 6-month follow up.

Some studies evaluated the use of lactobacilli for the prevention of recurrent UTI. Uehara *et al.* (2006) reported a study with nine patients with recurrent UTI. The patients were inserted with vaginal suppositories containing the strain *Lact. crispatus* GAI 98322 (1.0^8 CFU per suppository) every 2 days for 1 year. A significant reduction in the number of recurrences was noted, without any adverse complication. Czaja *et al.* (2007) performed a phase I trial to assess the safety and tolerance of a *Lactobacillus* vaginal suppository for prevention of recurrent UTI. Thirty premenopausal women with a

history of recurrent UTI were randomized to use *Lact. crispatus* CTV-05 or placebo vaginal suppositories daily for five days. No severe adverse events occurred. They concluded that *Lact. crispatus* CTV-05 can be given as a vaginal suppository with minimal side effects to healthy women with a history of recurrent UTI, but seven women randomized to *Lact. crispatus* CTV-05 developed pyuria without associated symptoms.

Beyond the vaginal application, probiotics can be administered orally to restore the vaginal microbiota.

Reid *et al.* (2004) performed a randomized, placebo-controlled trial; in that study 59 premenopausal women received either freeze-dried capsules (> 10^9CFU per capsule) containing *Lact. rhamnosus* GR-1 and *Lact. fermentum* RC-14 or calcium carbonate placebo by mouth, once daily for 60 days. After this time, the lactobacilli-dominant microbiota was restored in subjects with BV but not in controls. The mode of action is likely to be several-fold, in that in some subjects the probiotic strains ascend from the rectum to the vagina, while in other cases there is a reduction in ascension of pathogens, or an immune modulation that somehow displaces the BV organisms.

In the market, there are vaginal probiotic products that claim to restore or maintain the vaginal microbiota. For example, the tampon Ellen® (Ellen AB , Sweden) is sold with a mixture of three *Lactobacillus* spp. and a *P. acidilactici*. Vagiforte® (Bioflora, South Africa) is distributed in South Africa with *Lact. acidophilus, Bifidobacterium bifidum* and *Bifidobacterium longum* in vaginal suppositories. Gynoflor® (Gynoflor, Switzerland) are vaginal tablets which contain *Lact. acidophilus* and estriol.

1.7. *Pediococcus* spp. as bioprotective culture

Pediococcus spp. is a genus of Gram-positive lactic acid bacteria, placed within the family of *Lactobacillaceae*. *Pediococcus* spp. cells are spherical and arranged in tetrads, although, pairs are not uncommon in liquid cultures. They are facultative anaerobes, non-motile and non-sporulating.

The genus *Pediococcus* consists of the following species: *P. acidilactici*, *P. pentosaceus*, *P. damnosus*, *P. parvulus*, *P. inopinatus*, *P. halophilus*, *P. dextrinicus*, and *P. urinaeequi*. Various genetic tools have been used to discriminate between strains in the genus *Pediococcus* spp.. These include the use of specific DNA target probes, ribotyping, total DNA-DNA hybridization and 16S rDNA gene sequencing (Papagianni and Anastasiadou, 2009).

Pediococcus acidilactici and *P. pentosaceus* are mostly associated with food fermentations (Gurira and Buys, 2005) and have been used as starter cultures and probiotics in food (Jonganurakkun *et al.*, 2008; Vidhyasagar and Jeevaratnam, 2013).

The ability of *P. acidilactici* (Albano *et al.*, 2007; Anastasiadou *et al.*, 2008) and *P. pentosaceus* (Abrams *et al.*, 2011; Kingcha *et al.*, 2012) to produce antimicrobial peptides (bacteriocins) has been interesting for the use of the cultures or their products as protective cultures or biopreservatives, respectively.

The target of these bacteriocins, known as pediocins, is the cytoplasmic membrane of bacteria. These bacteriocins permeabilize the cytoplasmic membrane through pore formation, by insertion of the C-terminal regions into the membrane. This action includes loss of the permeability barrier and loss of the membrane potential, which, in strains that possess an autolytic system, results in cell lysis.

Bacteriocinogenic cultures, including pediococci, are marketed as protective cultures against common food spoilage bacteria and pathogens. Studies of bacteriocins for food

preservation are steadily increasing, but pediocins are also expected to find more applications in human and veterinary medicine (Papagianni and Anastasiadou, 2009).

1.8. Negative effects of probiotics

Annually, over one billion doses of probiotics are administered worldwide, and those administered for urogenital health have been well tolerated. Endocarditis and bacteraemia caused by lactobacilli are extremely rare; most cases occur in patients with chronic diseases or debilitating conditions, and any association with exposure to probiotic-containing foods or supplements has been questioned (Barrons and Tassone; 2008, Cribby *et al.*, 2008).

Nevertheless, safety of probiotic use must continually be monitored and considered when performing clinical studies. The potential for transfer of antibiotic resistance is one factor to consider, although it remains to be proven that probiotics have contributed in any way to drug resistance or disease (Cribby *et al.*, 2008).

2. Objectives of this study

The factors that affect and control the vaginal microbiota are still not fully understood, therefore the vaginal tract should be analyzed as a microbial ecosystem to understand the full range of factors that affect risk of disease.

The use of probiotics to colonize the vaginal tract and prevent or treat infection has been considered for some time and has an excellent safety record. It is recognized that LAB have the ability to maintain the vaginal health of women. Natural antimicrobials produced by probiotics, at the same time that inhibit the growth of pathogens, will improve the survival and promote the growth of the native vaginal microbiota, thus supporting the natural defenses against pathogenic microorganisms.

The selection and production of bioprotective cultures and probiotics in the food industry has been extensively studied and recognized.

The objective of this study was to select an isolate of LAB, belonging to one genus found in the normal vaginal microbiota (*Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp. or *Weisella* spp.) with antimicrobial activity against *L. monocytogenes*. The LAB selected can be used as a vaginal probiotic to prevent vaginal colonization of *L. monocytogenes* in pregnant women and thereby prevent neonatal listeriosis.

Specifically, it was intended to:

- Assess the behavior of *L. monocytogenes* in vaginal conditions
- Select an isolate of LAB that survives and inhibits *L. monocytogenes* in vaginal conditions
- Identify and characterize the selected strain of LAB
- Evaluate the antimicrobial mechanism
- Investigate a possible formulation for the administration of the isolated LAB

3. Survival and biofilm formation of *Listeria monocytogenes* in simulated vaginal fluid: influence of pH and strain origin

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3.1. Abstract

Listeria monocytogenes, the responsible agent for listeriosis, can be transmitted from mother to fetus/neonates by vertical transmission, transplacentally or during passage through the birth canal. The purpose of this study was to investigate the survival and biofilm formation of *L. monocytogenes* (isolated from clinical cases or from food) in simulated vaginal fluid at different pH values (4.2, 5.5 and 6.5). Results demonstrated that this pathogen is inhibited by the normal vaginal pH but may proliferate when it increases. Clinical strains were significantly more resistant to pH 4.2 than food isolates. All isolates tested were producers of biofilm at different pH values, however *L. monocytogenes* produced higher quantities of biofilm in nutrient-rich medium. No significant differences in biofilm production were detected between food and clinical isolates. *L. monocytogenes* survived and even grew at the higher pHs investigated, suggesting that fetus/neonates from women having increased vaginal pH values during pregnancy, may be at higher risk of listeriosis. As *L. monocytogenes* are biofilm producers, this increases the probability of occurrence of neonatal infection.

3.2. Introduction

The normal vaginal pH in pre-menopausal women varies between 3.5-4.5 (Boskey *et al.*, 1999; Donders *et al.*, 2007; Garcia-Closas *et al.*, 1999). Acid pH is due to the presence of *Lactobacillu*s spp. which are able to produce lactic acid from glycogen (Donati *et al.*, 2010). An increase of pH is caused by several factors resulting from a depletion of vaginal lactobacilli (Donders *et al.*, 2007; Simhan *et al.*, 2003), menstruation, unprotected sexual intercourse with deposition of semen (Tevi-Bénissan *et al.*, 1997) and vaginal medications. Elevated vaginal pH (5.0 to 6.5) can be associated with colonization by pathogenic microorganisms (Caillouette *et al.*, 1997; Pavletic *et al.*, 2004).

Vaginal infection during pregnancy represents many risks such as stillbirth, abortion, premature delivery or neonatal infection (Donati *et al.*, 2010).

Listeria monocytogenes, the responsible agent for listeriosis, is one of several microorganisms which can be transmitted from mother to fetus/neonate by vertical transmission, transplacentally or during passage through the birth canal (Delgado, 2008; Posfay-Barbe and Wald, 2009). *Listeria monocytogenes* can colonise the vagina by transperineal spread from the gastrointestinal tract, but it is difficult to prove the colonization of the gastrointestinal tract because this bacterium is fastidious and the normal flora is composed of a wide diversity of microorganisms. Therefore, screening of rectal or vaginal cultures is not useful in diagnosis of listerial infection (Posfay-Barbe and Wald, 2009).

Listeria monocytogenes in pregnancy can be asymptomatic or cause mild illness in women (such as fever, headache, diarrhea, myalgia), but may cause serious consequences to the neonate (Janakiraman, 2008). There are several serovars of *L.*

monocytogenes, although most of the documented human listeriosis is associated with serotypes 1/2a, 1/2b, 1/2c and 4b.

Neonatal listeriosis can be of early or late onset. In early onset, infection occurs *in utero* and the clinical manifestations appear 1.5 days after birth. The neonates present symptoms such as sepsis, respiratory distress, purulent conjunctivitis and skin lesions. In late onset, the symptoms arise several days or weeks after birth and the transmission occurs nosocomially or during delivery. In that case, neonates are more likely to have meningitis. This infection is acquired because some women carry *L. monocytogenes* in the vaginal tract (Delgado, 2008; Posfay-Barbe and Wald, 2009). However, according to some studies, the incidence of *L. monocytogenes* vaginal carriage is low (Stepanović *et al.*, 2007). They analysed 958 women of reproductive age and isolated only one strain of *L. monocytogenes* (an isolation rate of 0.1%), but this low rate was explained by rapid clearance of the bacteria from the genital tract. Although the incidence of *L. monocytogenes* in the vagina may be low, the consequences can be devastating.

Several studies demonstrated that *L. monocytogenes* is able to form biofilms (Bonaventura *et al.*, 2008; Harvey *et al.*, 2007; Stepanović *et al.*, 2004). Biofilms are microbial communities which adhere to surfaces by producing extracellular polymeric substances (Lindsay and von Holy, 2006). Biofilms are more resistant to antimicrobial agents, that contributes to the chronicity of infections (Stewart and Costerton, 2001). In addition, bacteria in biofilms are more difficult to isolate in culture, and sometimes the infections are not detected (Romero *et al.*, 2008).

The aim of this study was therefore to evaluate the survival and biofilm formation of *L. monocytogenes* in simulated vaginal fluid (SVF) at different pH values (normal and elevated vaginal pH).

3.3. Materials and methods

3.3.1. Microorganisms

In this study 20 isolates of *L. monocytogenes* of clinical (n = 10) and food origin (n = 10) were used (Table 3.1.). Stock cultures were maintained in Tryptone Soya broth with Yeast Extract (Lab M, Bury, United Kingdom) 0.6% w/v (TSB-YE) supplemented with 30% (w/v) of glycerol (Panreac, Barcelona, Spain) stored at -80 ºC. Cultures were grown in TSA-YE (Lab M) and were sub-cultured twice in TSB-YE at 37 ºC for 24 h.

Table 3.1. *Listeria monocytogenes* strains used in this study, indicating their serogroups and sources.

Isolate	Serogroup	Sample	Source
1796	IIb	Blood	clinical
2065	IIb	Blood	clinical
2076	IIb	Blood	clinical
1761	IIa	cerebrospinal fluid	clinical
2089	IVb	Unknow	clinical
2092	IVb	Blood	clinical
1762	IIb	Placenta	clinical
2096	IIa	Blood	clinical
2091	IIa	cerebrospinal fluid	clinical
2074	IVb	Blood	clinical
925/1	IIb	goat cheese	food
930/1	IIa	goat milk	food
1958/3	IIb	meat product	food
1308	IIc	fresh sausage	food
879/3	IIa	shrimp patties	food
1092	IVb	Sandwich	food
1055/4	IVb	Cheese	food
2485/2	IVb	Hotdog	food
FSL-F7-088	IIc	*alheira*[1]	food
FSL-F7-128	IIb	*alheira*[1]	food

[1] Portuguese traditional fermented sausage

3.3.2. Simulation of vaginal fluid

The composition of the simulating female genital tract secretions was described by Owen and Katz (1999): 3.51 g/L NaCl (Panreac); 1.40 g/L KOH (Pronalab, Lisbon,

Portugal); 0.222 g/L $Ca(OH)_2$ (Merck, Darmstadt, Germany); 0.018 g/L bovine serum albumin (Sigma-Aldrich, Steinheim, Germany); 2.00 g/L lactic acid (Fluka, Steinheim, Germany); 1.00 g/L acetic acid (Panreac); 0.16 g/L glycerol; 0.4 g/L urea (Sigma-Aldrich); 5.0 g/L glucose (Pronalab). Once these compounds were combined, the mixture was adjusted to a pH of 4.2, 5.5 and 6.5 using HCl (Pronalab) or NaOH (Pronalab).

3.3.3. Survival of *Listeria monocytogenes* in simulated vaginal fluid

The SVF was inoculated with 2% (v/v) of overnight culture of *L. monocytogenes* strains suspended in Ringer's solution (Oxoid, Hampshire, United Kingdom) with a final cell concentration of 10^5 CFU/mL and incubated at 37 ºC. Aliquots of 1 mL were withdrawn at defined intervals (2 h, 4 h, 6 h, 8 h, 24 h and 48 h) for further enumeration. Three independent replicates of these assays were performed. For each assay, controls in TSB at acidic and normal pH at 37 ºC were included.

The enumeration of *L. monocytogenes* was performed by serial 10-fold dilutions in Ringer's solution and samples were plated in duplicate on TSA-YE. Colony forming units per millilitre (CFU/mL) were determined after 24 h incubation at 37 ºC.

3.3.4. Biofilm assay

Quantification of biofilm production was performed as previously described by Stepanović *et al*. (2004), with some modifications. The wells of a sterile 96-well polystyrene microplate (Brand, Wertheim, Germany) were filled with 180 µL of TSB or SVF, both at pH 4.2, 5.5 and 6.5. A quantity of 20 µL of bacterial cells suspended in Ringer´s solution was added into each well. The plates were incubated aerobically for 24 h, 48 h and 72 h at 37 ºC. To quantify the biofilm formation, the wells were washed

three times with 250 µL of sterile distilled water. The attached bacteria were fixed with 200 µL of methanol (Romyl, Leics, United Kingdom) for 15 minutes and then microplates were emptied and dried at room temperature. Subsequently, 200 µL of a 2% v/v crystal violet solution (Merck) was added to each well and held at ambient temperature for 5 minutes. Excess stain was then removed by placing the plate under gently running tap water. Stain was released from adherent cells with 160 µL of 33% v/v glacial acetic acid. The optical density (OD) of each well was measured at 630 nm using a plate reader (Microplate reader, Bio-Rad, Hercules, CA, USA).

Each assay was tested nine times and the negative control was performed in TSB or SVF only (no cells of *L. monocytogenes*).

The cut-off (ODc) was defined as the mean OD value of the negative control. Based on the OD, strains were classified as: no biofilm producers ($OD \leq OD_C$), weak ($OD_C < OD \leq 2 \times OD_C$), moderate ($2 \times OD_C < OD \leq 4 \times OD_C$) or strong biofilm producers ($4 \times OD_C < OD$).

3.3.5. Statistical analysis

An analysis of variance (one-way ANOVA) was performed to test any significant effect of the pH and origin of strain (isolated from clinical cases or from food) on the survival and biofilm formation of *L. monocytogenes*. All calculations were carried out using the software Kaleidagraph (version 4.04, Synergy Software, Reading, USA).

3.4. Results

3.4.1 Survival of *Listeria monocytogenes* in simulated vaginal fluid

In this study, we simulated vaginal fluid to observe the behavior of *L. monocytogenes*. The results showed that survival of this bacterium is affected by pH ($p < 0.001$), being

that the acid pH of vaginal fluid inhibited *L. monocytogenes* (Figure 3.1.). In TSB at acidic pH no decrease in counts of *L. monocytogenes* at 48h was observed (data not shown).

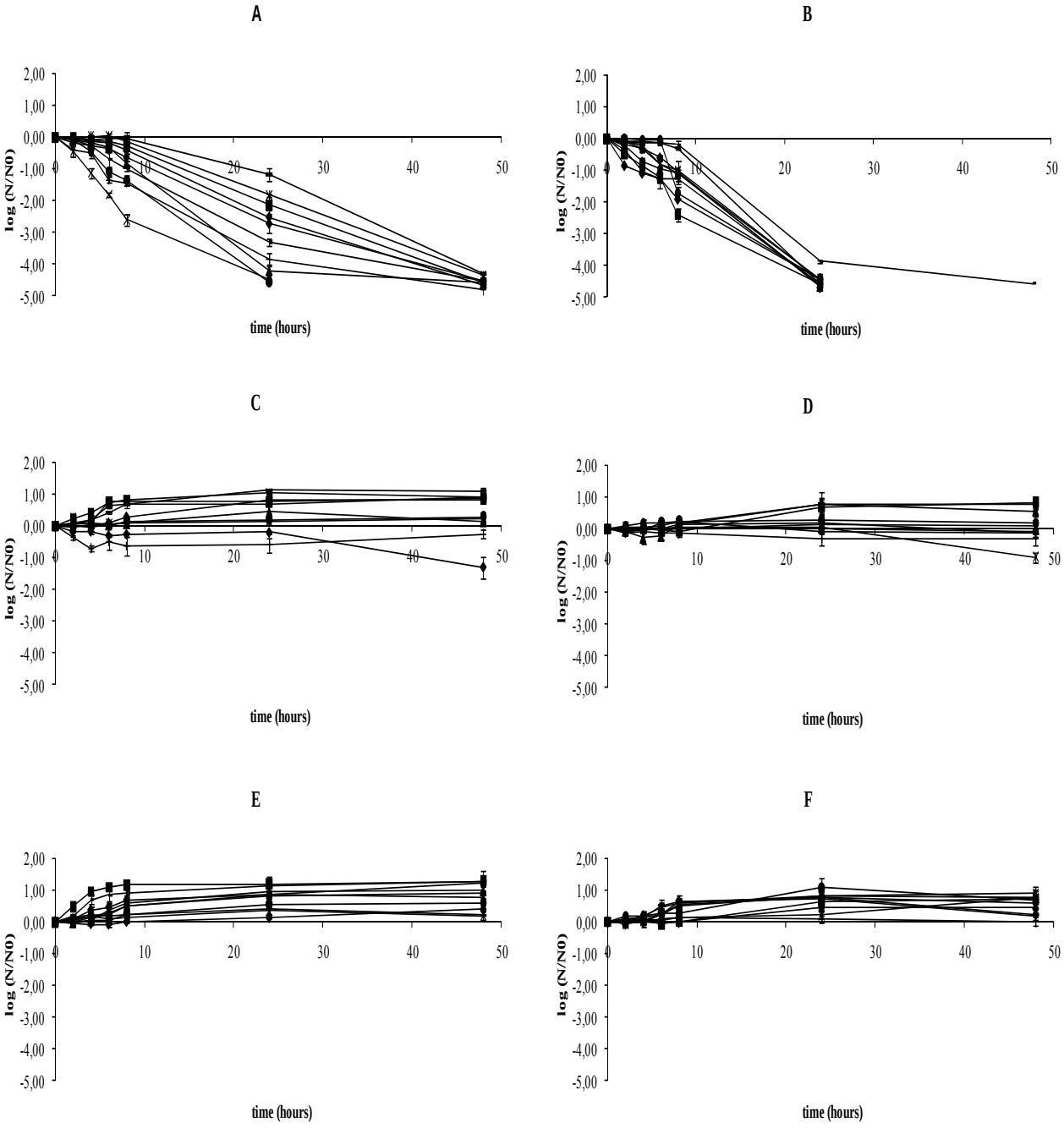

Figure 3.1. Survival of *L. monocytogenes* in simulated vaginal fluid. Clinical strains at: (A) pH = 4.2; (C) pH = 5.5; (E) pH = 6.5. Food strains at: (B) pH = 4.2; (D) pH = 5.5; (F) pH = 6.5. All points are means ± standard deviations and for each strain origin (clinical or food), the symbols of the correspondent graphs are the same for each isolate.

3.4.2. Biofilm assay

Quantification of biofilm showed that all strains of *L. monocytogenes* produced biofilm in TSB and SVF at different pH values. In vaginal fluid, strains were classified as weak or moderate producers; in TSB, *L. monocytogenes* were classified as weak, moderate or strong producers (Table 3.2.).

Table 3.2. Percentage of *L. monocytogenes* strains that produce biofilms in respective media at different time intervals, based on the optical densities strains were classified as strong, moderate or weak biofilm producers.

	Percentage of strains that produce biofilm (%)		
	Weak producer	Moderate producer	Strong producer
24 h			
Vaginal fluid at pH=4.2	70	30	0
Vaginal fluid at pH=5.5	95	5	0
Vaginal fluid at pH=6.5	55	45	0
TSB at pH=4.2	25	75	0
TSB at pH=5.5	10	55	35
TSB at pH=6.5	0	65	35
48 h			
Vaginal fluid at pH=4.2	35	65	0
Vaginal fluid at pH=5.5	30	70	0
Vaginal fluid at pH=6.5	45	55	0
TSB at pH=4.2	10	90	0
TSB at pH=5.5	5	45	50
TSB at pH=6.5	0	25	75
72 h			
Vaginal fluid at pH=4.2	25	75	0
Vaginal fluid at pH=5.5	55	45	0
Vaginal fluid at pH=6.5	35	65	0
TSB at pH=4.2	5	95	0
TSB at pH=5.5	0	30	70
TSB at pH=6.5	0	5	95

The quantity of biofilm produced was media dependent ($P < 0.001$). An example of biofilm formation by *L. monocytogenes* is shown in Figure 3.2.

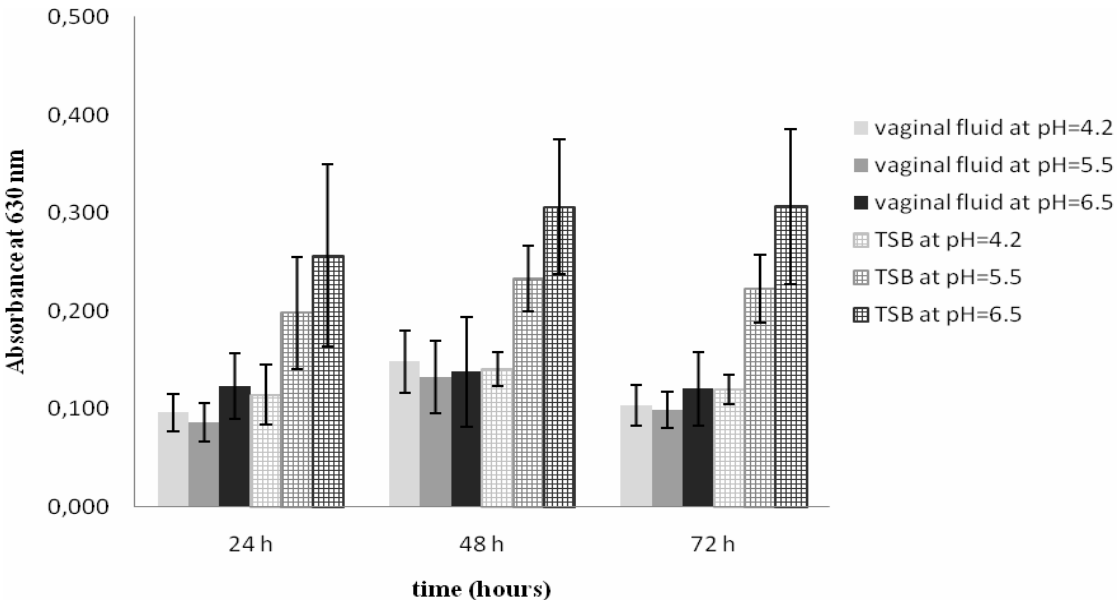

Figure 3.2. Production of biofilm by a representative isolate of *L. monocytogenes* (1761 IIa) at different time intervals, in SVF and TSB at different values of pH. Error bars represents mean ± standard deviation.

3.5. Discussion

This study demonstrates that survival of *L. monocytogenes* in SVF is pH dependent ($P < 0.001$), the pH of vaginal secretions influences the growth of *L. monocytogenes* and vaginal acidity inhibits this bacterium.

At pH of 4.2 there was a greater reduction in viable cells than at other pH values (Figures 3.1 A and B), thus demonstrating that in a normal vaginal pH, *L. monocytogenes* cannot grow. Mahmoud *et al*. (1995) studied the effect of vaginal fluid and pH in cultures of *C. trachomatis*. The investigation showed vaginal fluid decreased

the chlamydial infection and this inhibition was increased at a pH range of 3.5 and 4.5. Other investigations performed by Valore *et al*. (2002), showed that vaginal secretions with a high antimicrobial activity (against *Lact. jensenni*, GBS and *E. coli*) contained high levels of lactic acid.

Caillouette *et al*. (1997) demonstrated that vaginal pH is higher in women with colonization of pathogenic bacteria than in women with normal flora or yeast infections. Alkalization of vaginal fluid in pre-menopausal women may be indicative for the presence of pathogens (Caillouette *et al.*, 1997; Tevi-Bénissan *et al.*, 1997) and can be used in the diagnosis of vaginal infections.

To simulate vaginal infection, we used pH of 5.5 and 6.5 and found significant differences between these pH values. At pH 5.5 there was a reduction in some bacterial strains, but it was also demonstrated that some *L. monocytogenes* strains can grow at that pH (Figures 3.1. C and D).

At pH 6.5 all strains of *L. monocytogenes* were able to proliferate over time (Figures 3.1. E and 1 F). Therefore, colonization of *L. monocytogenes* is only possible when there is an increase in vaginal pH.

Geshnizgani and Onderdonk (1992), also developed a medium simulating *in vivo* conditions of vaginal fluid. However, to observe the growth of vaginal microorganisms (*Bacteroide fragilis, Propionibacterium jensenii, Lact. acidophilus, Veillonella parvula, Streptococcus intermedius, Corynebacterium and Staphylococcus epidermidis*), to levels consistent with those noted *in vivo*, the concentration for some components was modified.

At pH 4.2, *L. monocytogenes* of clinical origins were significantly more resistant (reduction of approximately 4.5 log after 48 h) than *L.monocytogenes* of food origin (reduction of approximately 4.5 log after 24 h) ($P < 0.001$). It has also been suggested

that clinical strains of *L. monocytogenes* are more resistant than food isolates to gastrointestinal tract conditions (Ramalheira *et al.*, 2010).

If *L. monocytogenes* can colonize the genital tract and grow when there is an increase of pH, in cases of pregnant women, this bacterium could be transmitted to the neonate by ascending to the uterus via or during passage of the fetus through the vagina. *L. monocytogenes* may cause significant morbidity and mortality in the neonates.

Evaluation of biofilm formation demonstrated that *L. monocytogenes* had the ability to form biofilm in TSB and SVF (Table 3.2.). However, there was more biofilm formation in TSB than in vaginal fluid ($P < 0.001$), so the medium composition influenced the quantity of biofilm production. These results confirm the conclusions of other studies, which demonstrated that the *L. monocytogenes* produced more biofilm in nutrient-rich media than nutrient-poor media (Hood and Zottola, 1997, Mai and Conner, 2007, Stepanović *et al.*, 2004).

In TSB medium, biofilm production was pH dependent ($P < 0.001$). This study showed that the *L. monocytogenes* produced less biofilm at pH of 4.2 than other pH values (5.5 and 6.5) after 24 h, 48 h and 72 h of incubation (Figure 3.2). The amount of cells attached increased with increasing of pH.

In SVF, at 24 h and 72 h we found significant differences in biofilm formation at different pH values ($P < 0.001$). However, no significant differences ($p > 0.5$) were observed at 48 h. The vaginal fluid used had a poor nutritional composition, this stress can overrule the stress caused by the pH values. Therefore, the effect of pH was not evident when using the SVF (Figure 3.2.).

In this study, we also observed that *L. monocytogenes* strains differ in the quantity of biofilm produced ($P < 0.001$), however no significant differences were observed for strains from different sources (food and clinical). These results were similar to those

found in previous studies, which did not find a correlation between several sources (human, animal and food), but also found statistical differences between strains of *L. monocytogenes* (Chae and Schraft, 2000; Stepanović *et al.*, 2004).

According to Romero *et al*. (2008), several microorganisms can form biofilm in the amniotic cavity, so it will be important to characterize these organisms. As *L. monocytogenes* can produce biofilm, infections caused by this bacterium may be underestimated because the diagnoses in the presence of biofilm are difficult and some techniques are inadequate.

4. Survival and biofilm formation by Group B streptococci in simulated vaginal fluid at different pHs

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4.1. Abstract

The GBS is an important cause of neonatal and maternal infection. Group B *Streptococcus* is a commensal organism of the lower gastrointestinal and vaginal tract. A frequent mode of neonatal infection is vertical transmission from pregnant women to their foetus or neonate. The aim of this study was to evaluate the survival and biofilm production of 10 GBS strains in SVF at pH 4.2, 5.5 and 6.5. Group B *Streptococcus* survived longer at higher pH than at normal vaginal pH. At pH 4.2, with the exception of two isolates that were recovered up to 48 h and 72 h, viable GBS numbers declined below the limit of detection by 24 h. At higher pH, GBS survived between 3 and 15 days. All isolates investigated were biofilm producers but biofilm production was greater in TSB compared to SVF. The quantity of biofilm produced increased with the rise in the pH. This study suggests that high vaginal pH may influence both GBS survival and biofilm production and thus could be a risk factor for GBS infection.

4.2. Introduction

Group B *Streptococcus* is an important cause of neonatal and maternal infection world-wide and is responsible for significant neonatal morbidity and mortality. In the 1970s, GBS was the leading cause of neonatal infections, with a mortality rate between 20% and 50% in cases of early-onset disease (Gibbs *et al.*, 2004). In the United States the incidence of GBS in the 1990s was 1.7/1000 live births. Following the implementation of intrapartum prophylaxis to prevent GBS disease the incidence decreased to 0.34-0.37/1000 live births (Verani *et al.*, 2010).

Between 2001 and 2005, the incidence of GBS infection in Portugal was 0.54/1000 live births, of which 81% occurred during the first week after birth. The mortality rate was 6.6%, with preterm infants the most affected (Neto, 2008).

Group B *Streptococcus* infection is frequent in the first trimester of pregnancy but usually is asymptomatic in pregnant women. However, clinical manifestations can be diverse and serious, including urinary tract infection, amnionitis, puerperal sepsis, meningitis, endocarditis or other complications. Group B *Streptococcus* genital colonization, in some instances, may be associated with preterm delivery, preterm premature rupture of membranes and low birth weight (Fernandez and Carol, 1999; Gibbs *et al.*, 2004; Jeffery, 1996).

The genital and gastrointestinal tracts of pregnant women are the most important sources of GBS; colonization can be intermittent, transient or chronic (Gibbs *et al.*, 2004; Puopolo, 2008). The usual mode of neonatal infection by GBS is vertical transmission from pregnant women to their foetus (by ascending through the amniotic membranes) or to neonates by contact with GBS in the birth canal during labour (Puopolo, 2008). Group B *Streptococcus* maternal colonization and vertical

transmission is likely responsible for more than 95% of neonatal carriers (Spellerberg, 2000).

The development of early-onset disease occurs by vertical transmission and clinical manifestations (septicemia, pneumonia or meningitis) are evident during the first 6 days of life. Late-onset disease may be acquired by vertical or horizontal transmission (maternal, family and nosocomial sources) and infection manifests between 7 and 90 days of age. The most common presentations of late-onset diseases are meningitis and septicemia without a focus (Fernandez and Carol, 1999; Gibbs *et al.*, 2004; Jeffery, 1996).

Biofilm formation may have an important role in the pathogenicity of GBS (Kaur *et al.*, 2009; Konto-Ghiorghi *et al.*, 2009; Rinaudo *et al.*, 2010). The purpose of this study was to assess the survival and biofilm formation of GBS in simulated vaginal conditions.

4.3. Materials and methods

4.3.1. Microorganisms and growth conditions

Ten isolates of GBS recovered from asymptomatic pregnant women were kindly provided by Hospital S. Marcos (Braga). Stock cultures were maintained in TSB supplemented with 30% (v/v) of glycerol stored at -80 ºC. Working cultures of GBS were grown on TSA and were sub-cultured in TSB at 37 ºC for 24 h. Overnight cultures of GBS were centrifuged at 8877 x g at 5 min (Rotina 35; Hettich Zentrigugen, Oxford, CT) and the cell pellet was resuspended in Ringer's solution.

4.3.2 Simulated vaginal fluid

Simulated vaginal fluid was prepared according to Owen and Katz (1999) (Table 4.1.). This mixture was adjusted to pH 4.2, 5.5 and 6.5 using HCl or NaOH. These pH values

were used to reproduce the vaginal pH in normal conditions (pH 4.2) and in case of infection (pH 5.5 and 6.5) (Caillouette *et al.*, 1997; Pavletic *et al.*, 2004).

Table 4.1. Composition of simulated vaginal fluid (SVF).

Component	Concentration (g/L)
NaCl	3.51
KOH	1.40
$Ca(OH)_2$	0.222
Bovine serum albumin	0.018
Lactic acid	2.00
Acetic acid	1.00
Glycerol	0.16
Urea	0.4
Glucose	5.0

4.3.3 Survival of Group B streptococci in simulated vaginal fluid

Survival of GBS in vaginal fluid was investigated according to Borges *et al*. (2011). The SVF was inoculated with 2% (v/v) of harvested cultures with a final cell concentration of 10^5 CFU/mL and incubated at 37 ºC. At various time intervals, aliquots were withdrawn for further enumeration. Three independent replicates were performed and controls in TSB at different pH (4.2, 5.5 and 6.5) at 37 ºC were included (data not shown). The enumeration of GBS was performed by serial 10-fold dilutions in Ringer's solution and samples were plated in duplicated on TSA. Colony forming units per millilitre (CFU/mL) were determined after 24h of incubation at 37 ºC. Results were expressed as log (N/N_0), where N represents the CFU/mL of GBS over time and N_0 represents the CFU/mL at the initial time.

4.3.4 Biofilm assay

Quantification of biofilm production was performed according to Borges *et al.* (2011). The wells of a sterile 96-well polystyrene microplate were filled with 180 µL of TSB or SVF (pH 4.2, 5.5 and 6.5) and 20 µl of bacterial cell suspension and incubated aerobically for 24 h, 48 h and 72 h at 37 ºC. Biofilm formation was quantified washing the wells three times with 250 µL of sterile distilled water. The attached bacteria were fixed with 200 µL of methanol for 15 minutes and then microplates were emptied and dried at room temperature. Subsequently, 200 µL of a 2% (v/v) crystal violet solution was added to each well and held at ambient temperature for 5 minutes. Excess stain was then removed by placing the plate under gently running tap water. Stain was released from adherent cells with 160 µL of 33% (v/v) glacial acetic acid. The OD of each well was measured at 630 nm using a microplate reader. Triplicate assays, repeated three times were performed. Uninoculated TSB or SVF were used as negative controls. The cut-off (ODc) was defined as the mean OD value of the negative control. Based on the OD, strains were classified as: no biofilm producers ($OD \leq OD_C$), weak ($OD_C < OD \leq 2 \times OD_C$), moderate ($2 \times OD_C < OD \leq 4 \times OD_C$) or strong biofilm producers ($4 \times OD_C < OD$) (Figure 4.1.).

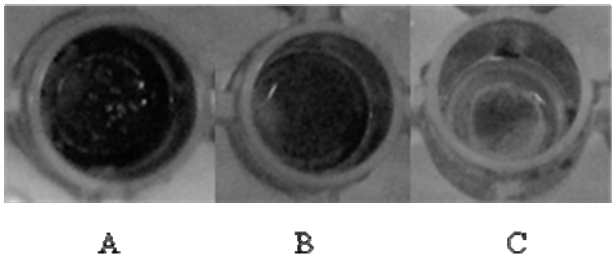

Figure 4.1. Biofilm stained with crystal violet. (A) strong producer; (B) moderate producer; (C) weak producer.

4.3.5. Statistical analysis

An ANOVA was performed to test any significant effect of the pH, media and strain on the survival and biofilm formation of GBS. All calculations were carried out using the software Kaleidagraph.

4.4. Results

4.4.1 Survival of Group B streptococci in simulated vaginal fluid

The survival of GBS in vaginal fluid was significantly lower ($P < 0.001$) at pH 4.2 than at higher pH (5.5 and 6.5): data for representative strains are shown in Figure 4.2. At normal vaginal pH, culturable GBS levels declined to below the detection limit of the enumeration technique (< 1.4 log CFU/mL) in 24 h, with the exception of two isolates that were detected up to 48 h and 72 h. As no increase in GBS CFU/mL was detected at even the earliest time points (< 24 h), this suggests a failure of these GBS strains to grow in SVF. At pH 5.5 and 6.5, GBS strains demonstrated a similar ($P > 0.05$) behavior and survived for longer periods (3-15 days) than at pH 4.2. Survival was demonstrated to be strain dependent ($P < 0.005$).

The pH was measured over the time course of the experiment and no significant differences were observed (data not shown). Again these data suggest an absence of GBS growth as no acidification of the media due to glucose fermentation was observed.

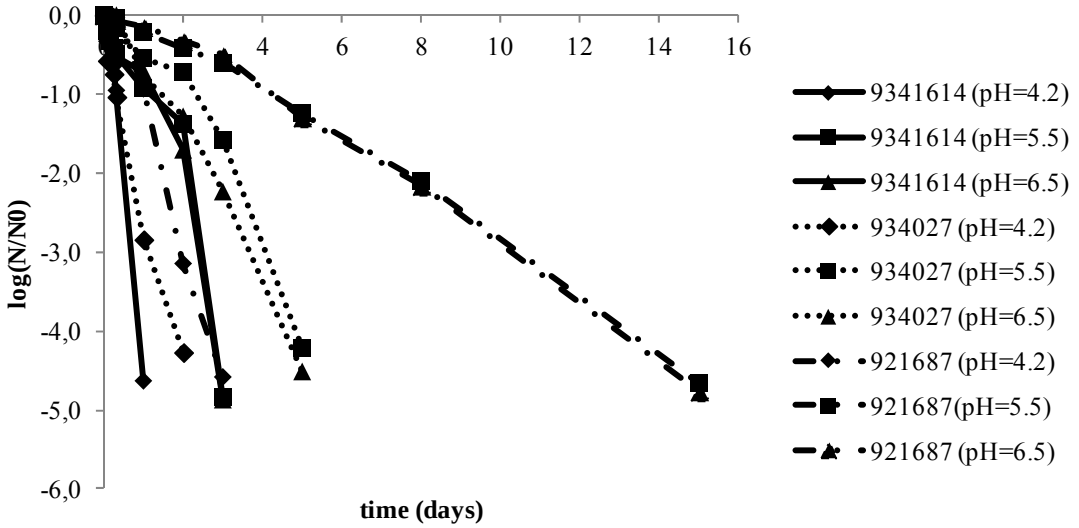

Figure 4.2. Survival of three representative GBS strains in SVF at different pH: (━) strain more sensitive; (···) strain with intermediate survival; (━ ·) strain more resistant. Data represented as the mean of the log (N/N_0) ± standard deviation.

4.4.2 Biofilm assay

All the GBS isolates studies were biofilm producers in SVF and TSB. The biofilm production was strain dependent ($P < 0.001$). Figure 4.3. presents a representative example of biofilm formation by one isolate of GBS (isolate 933043) in different media, at different values of pH and at different time intervals (24 h, 48 h, 72 h).

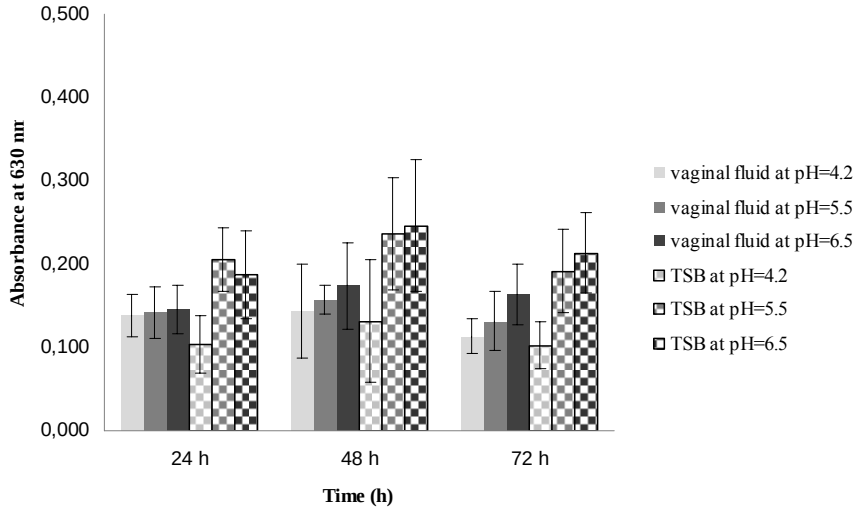

Figure 4.3. Production of biofilm by a representative isolate of GBS at different time intervals, in SVF and TSB at different values of pH. Error bars represent standard deviation of the mean.

The production of biofilms in SVF and in TSB was significantly different ($P < 0.001$); GBS strains formed greater quantities of biofilm in TSB than in SVF. In SVF the GBS isolates were classified as weak and moderate producers, whereas in TSB the isolates were classified as moderate and strong producers (Table 4.2).

In SVF, no significant differences ($P = 0.15$) were observed in biofilm formation at pH 4.2 and 5.5 whereas at pH 6.5 more biofilm production was observed ($P < 0.05$).

No significant differences ($P = 0.90$) were observed in the production of biofilm in TSB at pH 5.5 and 6.5, whereas at pH 4.2 the production of biofilm was significantly lower ($P < 0.001$).

No significant differences ($P = 0.95$) in the biofilm production were detected at 24 h and 72 h for either SVF or TSB. In comparison, Kaur *et al*. (2009) reported that maximum biofilm formation by GBS was obtained at 48 h, comparing with 24 h, 72 h and 96 h.

Table 4.2. Isolates of GBS that produce biofilm in respective media at different time intervals. Based on the optical densities strains were classified as strong, moderate or weak biofilm producers.

	Strains that produce biofilm (n = 10)		
	Weak producer	Moderate producer	Strong producer
24 h			
Vaginal fluid at pH=4.2	3	7	0
Vaginal fluid at pH=5.5	2	8	0
Vaginal fluid at pH=6.5	2	8	0
TSB at pH=4.2	0	9	1
TSB at pH=5.5	0	7	3
TSB at pH=6.5	0	6	4
48 h			
Vaginal fluid at pH=4.2	2	8	0
Vaginal fluid at pH=5.5	2	8	0
Vaginal fluid at pH=6.5	3	7	0
TSB at pH=4.2	0	9	1
TSB at pH=5.5	0	7	3
TSB at pH=6.5	0	6	4
72 h			
Vaginal fluid at pH=4.2	3	7	0
Vaginal fluid at pH=5.5	3	7	0
Vaginal fluid at pH=6.5	3	6	1
TSB at pH=4.2	0	9	1
TSB at pH=5.5	1	5	4
TSB at pH=6.5	0	6	4

4.5. Discussion

Infections in the genital tract can affect pregnant women. These infections cause premature birth, neonatal infection, congenital abnormalities and morbidity in the mother (Hay, 2005). Therefore, it is important to understand the behavior of pathogens in vaginal conditions in order to understand their pathogenesis and ascending infection. Caillouette *et al*. (1997) reported an association between vaginal pH and the presence of pathogens. In that study, vaginal pH and aerobic and yeast cultures of 55 premenopausal women were evaluated. Patients carrying potentially pathogenic bacteria (including GBS) had a higher vaginal pH than the patients with normal vaginal microbiota or yeast infection. Hence, in premenopausal women a normal vaginal pH of ≤ 4.5 could be an indicator of the absence of bacterial pathogens, an elevated pH (range of 5.0 to 6.5) may suggest their presence.

In the present study, at pH 4.2 most of the GBS isolates were no longer viable within 24 h, whereas at pH 5.5 and 6.5 the GBS survived longer. These results are in agreement with those reported in a study on the survival of *L. monocytogenes* in vaginal fluid (Borges *et al.*, 2011), which concluded that at pH 4.2 a greater reduction in viable cells was observed than at pH 5.5 and 6.5.

Group B *Streptococcus* recurrently colonize the vaginal tract, often in conditions of altered microbiota such as BV (Locksmith and Duff, 2001). In BV there is an increased of vaginal pH because *Lactobacillus* spp. are replaced by anaerobic bacteria (Moodley and Sturm, 2000; Simhan *et al.*, 2003), perhaps enabling the colonization of GBS. Thus, a rise in vaginal pH may contribute to extended survival of colonising GBS.

The capacity of biofilm formation is another important virulence determinant for pathogenesis in humans. Previous studies reported that GBS are biofilm producers (Kaur *et al.*, 2009; Konto-Ghiorghi *et al.*; 2009, Rinaudo *et al.*, 2010), as was also

demonstrated in the present study. Here it was found that GBS produced a greater quantity of biofilm in TSB than in SVF. *Listeria monocytogenes* exhibited a similar behavior, producing more quantity of biofilm in TSB than in SVF (Borges *et al.*, 2011). In both media (SVF and TSB) it was observed that biofilm production was pH dependent; GBS formed more biofilm at pH 6.5 than at pH 4.2. These results were similar to those found in a study by Kaur *et al.* (2009), in which biofilm formation by GBS in TSB varied with pH and the amount of cells attached increased with the increase of pH. Rinaudo *et al.* (2010) reported that biofilm formation by GBS strain 515 (serotype Ia) in Todd Hewitt broth was observed only in the presence of glucose or sucrose (≥ 0.4% w/v) whilst Konto-Ghiorghi *et al.* (2009) reported that biofilm formation by GBS strain NEM316 (serotype III) on polystyrene was reduced in nutritionally rich media (such as Todd Hewitt) compared to weaker media (such as Roswell Park Memorial Institute medium) although the latter also had to be supplemented with 1% glucose. Variation in GBS biofilm forming capability in part reflects expression of particular pili (Konto-Ghiorghi *et al.*, 2009; Rinaudo *et al.*, 2010). Clearly biofilm formation by GBS strains is highly strain and medium dependent.

In conclusion, this study suggests that increasing vaginal pH may influence both GBS survival and biofilm production and thereby could be a risk factor for GBS infection.

5. Evaluation of characteristics of *Pediococcus* spp. to be used as a vaginal probiotic

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5.1. Abstract

Aims: The aim of our research was to select, identify and characterize an isolate of lactic acid bacteria to be considered as a vaginal probiotic.

Methods and Results: Thirty-five isolates of *Pediococcus* spp. showed bacteriocinogenic activity against *L. monocytogenes* and the ability to survive in simulated vaginal fluid at pH 4.2. One isolate of *Pediococcus* spp. was selected and characterized to evaluate its safety before use as vaginal probiotic. *Pediococcus pentosaceus* SB83 did not show the presence of virulence factors such as the production of gelatinase, lipase and DNase, hemolytic activity, nor the presence of virulence genes (genes *esp*, *agg*, *gel*E, *efaAfm*, *efaAfs*, *cylA*, *cylB* and c*ylM*).

Pediococcus pentosaceus SB83 was considered sensitive to cloramphenicol, gentamicin, streptomycin, kanamycin, erythromycin, and ampicillin. This strain was considered resistant to tetracycline and vancomycin.

Pediococcus pentosaceus SB83 was a biofilm-producer at different pH values (4.2, 5.5 and 6.5) in simulated vaginal fluid and in De Man, Rogosa and Sharpe medium.

Conclusions: The *in vitro* results provide a basis for the use of *P. pentosaceus* SB83 as a vaginal probiotic, to prevent colonization by *L. monocytogenes* in pregnant women.

Significance and Impact of Study: The application of vaginal probiotics could have the potential for preventing vaginal infections and consequently reduce abortion and neonatal infections.

5.2. Introduction

The normal vaginal microbiota is dominated by lactobacilli, at 10^7 to 10^8 CFU/mL of vaginal fluid in healthy premenopausal women (Bolton *et al.*, 2008; Boris and Barbés, 2000; Dicks *et al.*, 2000). Lactobacilli contribute to the prevention of genital infections and play a role in the maintenance of a healthy state. The capacity that lactobacilli have to adhere and compete for adhesion sites in the vaginal epithelium (Coudeyras *et al.*, 2008; Zárate and Nader-Macias, 2006); and the capacity to produce antimicrobial compounds e.g. hydrogen peroxide (Aslim and Kilic, 2006; Wasiela *et al.*, 2008), lactic acid (Fraga *et al.*, 2008), bacteriocin-like substances (Aroutcheva *et al.*, 2001; Dover *et al.*, 2007; Turovskiy *et al.*, 2009) and biosurfactants, are important in the impairment of colonization by pathogens. Furthermore, the production of lactic acid may help to maintain a low vaginal pH, approximately 4-4.5, which makes the vaginal environment more conducive to lactobacilli growth (Bolton *et al.*, 2008; Boris and Barbés, 2000; Reid and Bocking, 2003). Low vaginal pH is also beneficial for other antimicrobials; H_2O_2 is stable in these conditions and bacteriocins are highly active. An increase of the vaginal pH leads to a decrease in the lactobacilli-associated antimicrobial activity (Dover *et al.*, 2008).

Maternal urinary tract infections increase the risk of puerperal sepsis and the associated risk of neonatal sepsis (Ganatra and Zaidi, 2010). In pregnancy, infections can be transmitted from mother to child causing adverse sequelae, such as abortion, stillbirth, neonatal infection, etc. Infections of the newborn may be acquired in utero (congenital), around the time of delivery (intrapartum infection) or in the neonatal period (postpartum infection).

There is often a loss of colonization by lactobacilli due to antibiotic therapy, douching, sexual activity, hormone deficiency, and contraceptive measures (Barrons and Tassone,

2008; Bolton *et al.*, 2008). The selection and use of "vaginal probiotics" can be important to restore a healthy vaginal microbiota (Pascual *et al.*, 2008b).

Probiotics have been defined as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" (World Health Organization, 2005). Lactic acid bacteria may play a major role in preventing illness of the host, including bacterial vaginosis, yeast vaginitis, urinary tract infection and sexually transmitted diseases. The administration of probiotics by mouth and intravaginally has been shown to be safe, and for pregnant women this restoration could be important to lower the risk of pre-term labour (Reid and Bocking, 2003; Wilks *et al.*, 2004). The capacity of LAB to colonize the vaginal mucosa depends on the route of delivery; oral formulations must be capable of maintaining their structural integrity (viability) during passage through the gastrointestinal tract and delivery to the rectal area for ascension and colonization of the vaginal tract (Barrons and Tassone, 2008).

There are more than 80 known species of lactobacilli in the intestines and vagina, therefore, in terms of probiotic effects, individual species may differ in their ability to restore normal microbiota and regulate the overgrowth of pathogens (Barrons and Tassone, 2008).

Vaginal colonization by pathogens can result in transmission to the fetus/neonate by vertical transmission. Therefore, the vaginal application of LAB could be a preventative strategy to reduce the global burden of neonatal infections.

The aim of this study was to select, identify and characterize an isolate of LAB to be considered as a vaginal probiotic candidate.

5.3. Materials and methods

5.3.1. Microorganisms

5.3.1.1. Lactic acid bacteria isolates

In this study, fifty six food isolates (Escola Superior de Biotecnologia culture collection) and 19 vaginal isolates (Hospital São Marcos, Braga, Portugal) of LAB were selected for study. Stock cultures were maintained in de Man, Rogosa and Sharpe (MRS) broth (Lab M) supplemented with 30% (v/v) of glycerol and stored at -80 ºC. Cultures were recovered on MRS agar (Lab M) and were sub-cultured twice in MRS broth at 37 ºC for 24 h before use in all tests.

5.3.1.2. Pathogenic strains

As target bacteria for the inhibitory effects of LAB, 29 clinical isolates of *L. monocytogenes* from the *Listeria* culture collection of Escola Superior de Biotecnologia (Table 5.1.) and 10 vaginal isolates of GBS (Hospital São Marcos, Braga) were used. Cultures were grown on TSA-YE and were sub-cultured in TSB-YE at 37 ºC for 24 h. Stock cultures were maintained in TSB-YE containing 30% (v/v) of glycerol and stored at -80 ºC.

More than 95% of infections by *L. monocytogenes* in humans are caused by serotypes 1/2a, 1/2b and 4b and the majority of listeriosis outbreaks are caused by strains of serotype 4b (Swaminathan and Gerner-Smidt, 2007). Therefore, in this study we used clinical isolates of *L. monocytogenes* of these three different serotypes.

Table 5.1. *Listeria monocytogenes* isolates used in this study.

Isolate	Serotype	Isolated from
866	4b	Blood
999	1/2b	cerebrospinal fluid
1002	1/2b	Blood
1062	1/2b	Blood
1198	1/2a	Blood
1562/A	1/2b	Blood
1562/B	1/2b	Blood
1761	1/2a	cerebrospinal fluid
1762	1/2b	Placenta
1796	1/2b	Blood
1891	1/2a	cerebrospinal fluid
2065	1/2b	Blood
2074	4b	blood (mother and neonate)
2076	1/2b	Blood
2085/1	4b	cerebrospinal fluid
2085/2	4b	cerebrospinal fluid
2087	4b	Blood
2088	4b	Blood
2089	4b	Unknow
2091	1/2a	cerebrospinal fluid
2092	4b	Blood
2096	1/2a	Blood
2097	4b	Blood
2099	1/2a	cerebrospinal fluid
2100	4b	Blood
2101	4b	Blood
2103	1/2a	Blood
2135	1/2a	Blood
2141	4b	Blood

5.3.2. Screening for antimicrobial activity of lactic acid bacteria

The antimicrobial activities of LAB isolates were tested against pathogenic bacteria by using the agar-spot test method. TSA-YE plates were spread with overnight suspensions of each of the target bacteria and drops (10 μL) of overnight cultures of the LAB isolates were spotted on the lawns of pathogens (*L. monocytogenes* and GBS) and incubated for 24 h at 37 ºC. Translucent halo zones observed around the spots were

registered as positive for antimicrobial activity. For the positive strains, in order to determine the nature of inhibition, the pH of the supernatant, obtained by centrifugation at 8877 x g, 10 min, 4 ºC , was adjusted to 6 with sterile 1 M NaOH (Pronalab, Lisbon, Portugal) and then treated with catalase (0.1 mg/mL; Sigma-Aldrich) and trypsin (0.1 mg/mL; Sigma-Aldrich). After each of these treatments, supernatants were spotted against target bacteria (*L. monocytogenes* and GBS). As a control *P. acidilactici* HA-6111-2 was used (Albano *et al.*, 2007).

5.3.3. Quantification of antibacterial activity

For those LAB strains that showed antimicrobial activity by bacteriocin production (i.e. no reduction in activity after pH adjustment and treatment with catalase, but total loss of activity after trypsin treatment), this activity was quantified. Antimicrobial activity was expressed as arbitrary units (AU) per mL. AU is defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition of the target strains (Van Reenen *et al.*, 1998). Six isolates of *L. monocytogenes* of different serotypes (1/2b, 4b and 1/2a) were used as target strains.

5.3.4. Identification of bacteriocin-producing lactic acid bacteria

Total (genomic and plasmid) DNA isolation was performed using a Gen Elute Bacterial Genomic DNA kit (Sigma-Aldrich). Amplification of the 16S rDNA was carried out with the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') (MWG Biotech AG, Ebersberg, Germany) and 1492R (5'-GGTTACCTTGTTACGACTT-3') (MWG Biotech AG). Amplification reactions were performed in a Thermocycler (MyCycler™, Thermocycler Firmware, Bio-Rad, Richmond, CA) as follows: initial denaturation at 95 ºC for 5 min, 30 cycles of 1 min at 94 ºC, and 1 min at 55 ºC, followed by an increase to

72 ºC over 1.5 min. Extension of the amplified product was at 72 ºC for 10 min. Following amplification, 5 µL of product was separated at 90 V for 50 min in a 1% (w/v) agarose gel in 1x TAE buffer (4.84 g Tris-base, 1.09 g glacial acetic acid, 0.29 g ethylenediaminetetraacetic acid, 1 l distilled water), and then stained with 0.5 µg/mL of ethidium bromide. A 100-bp DNA ladder (Bio-Rad) was used as a molecular weight marker. PCR products, used as templates, were then purified with the GFX PCR DNA and Band Purification kit (GE HealthCare, Amersham Biosciences, Amersham, UK) and sent for sequencing. Sequences obtained were aligned with sequences in GenBank using the BLAST program.

5.3.5. Random amplified polymorphic DNA (RAPD)

The genetic heterogeneity of isolates was determined by numerical analysis of DNA profiles obtained by RAPD-PCR.

DNA primers M13 (5'-GAG GGT GGC GGT TCT-3') (MWG Biotech AG) and D8635 (5'-GAG CGG CCA AAG GGA GCA GAC-3') (MWG Biotech AG) were used. RAPD-PCR was performed on total DNA, according to Andrighetto *et al.* (2001). The 25 µL reaction volume composed of the primer M13: 0.99 pM primer, 150 mM of dNTPs (ABGene, Surrey, United Kingdom), 1 x PCR buffer (MBI Fermentas, Mundolsheim, France), 2.5 mM of $MgCl_2$ (MBI Fermentas), 2 U of Taq DNA polymerase (MBI Fermentas) and 1 µL of extracted solution of DNA. For the primer D8635 the mixture contained 0.88 pM of primer, 200 mM mM of dNTPs, 1 x PCR buffer, 2.5 mM of $MgCl_2$, 2 U Taq polymerase and 1 µL of extracted solution of DNA.

Amplification was performed in a Thermocycler by using the following program: initial denaturation at 94 ºC for 2 min, 35 cycles of 1 min per cycle at 94 ºC, annealing temperature of 46.9 ºC for 1 min, followed by an increase to 72 ºC over 1.5 min. Extension of the amplified product was at 72 ºC for 10 min. Amplified products were

separated by electrophoresis in 1.2% (w/v) agarose gels in 1x TAE buffer at 80 V for 2 h. Gels were stained in TAE buffer containing 0.5 μg/mL of ethidium bromide. A 100 bp DNA ladder was used as molecular weight marker.

The gels were photographed on a UV transilluminator (GelDoc2000, Bio-Rad) and image analysis was accomplished using Quantity One® software (Bio-Rad).

RAPD-PCR profiles were subsequently analyzed using the Gel Compar II Software (Applied Maths, Kortrijk, Belgium). Calculation of the similarity of the bands profile and grouping of the RAPD-PCR patterns were based on the Pearson's coefficient and agglomerative clustering was performed with the unweighted pairs group matching algorithm (UPGMA).

5.3.6. Survival of lactic acid bacteria in simulated vaginal fluid

For LAB strains that showed antimicrobial activity, survival in SVF was investigated according to Borges *et al.* (2011). Simulated vaginal fluid was prepared using components described in Table 5.2. This mixture was adjusted to a pH of 4.2 using HCl. Lactic acid bacteria cells were harvested by centrifugation at 8877 x g for 10 min at 4 ºC and washed twice in sterile Ringer's solution. Pellets were then re-suspended to the original volume in Ringer's solution.

The SVF was inoculated with 2% (v/v) of harvested cultures of LAB, with a final cell density of 10^7 CFU/mL and incubated at 37 ºC. At various time intervals (0 h, 4 h, 8 h, 24 h, 48 h), aliquots were withdrawn for further enumeration. Two independent replicates of these assays were performed. The enumeration of LAB was performed by serial 10-fold dilutions in Ringer's solution and 20 μL were spread-plated in duplicate on MRS agar. Colony forming units per millilitre (CFU/mL) were determined after 48 h

of incubation at 37 ºC. Results were expressed as log (N/N_0), where N represents the CFU mL of LAB at times after inoculation, and N_0 represents the initial CFU/mL. Simultaneously, 1 mL of each LAB culture was added to 1 mL of SVF. After 24 h of contact, antimicrobial activity was tested against six isolates of *L. monocytogenes* of different serotypes (1/2b, 4b and 1/2a), using the agar-spot test method (previously described). Translucent halo zones observed around the spots were registered as positive for antimicrobial activity. As a negative control, SVF (without LAB cells) was used.

Table 5.2. Composition of SVF (Owen and Katz 1999).

Component	Concentration (g/L)
NaCl	3.5
KOH	1.4
$Ca(OH)_2$	0.22
Bovine serum albumin	0.018
Lactic acid	2.0
Acetic acid	1.0
Glycerol	0.16
Urea	0.40
Glucose	5.0

5.3.7. Characterization of *Pediococcus pentosaceus* SB83

Based on the bacteriocinogenic activity and survival in SVF, one isolate of LAB (*P. pentosaceus* SB83) was selected for further studies.

5.3.7.1 Production of hydrolytic enzymes

The gelatinase and lipase activity were assessed according to Tiago *et al.* (2004). DNase activity was tested as described by Ben Omar *et al.* (2004).

All experiments were performed in duplicate and *Staphylococcus aureus* ATCC 25213 was used as a positive control.

5.3.7.2. Hemolytic activity

Production of hemolysin was determined by streaking *P. pentosaceus* SB83 on a plate of Columbia agar with 5% of sheep blood (BioMérieux, Marcy l'Etoile, France). Plates were incubated at 37 ºC for 24 h, under aerobic conditions, after which plates were examined for hemolysis. *Ent. faecalis* F2 (from a collection of Tracy Eaton, Division of Food Safety Sciences, Institute of Food Research, Norwich, United Kingdom) and *Ent. faecalis* DS16 (from a collection of C. B. Clewell, Department of Oral Biology, School of Dentistry, University of Michigan, Ann Arbor, USA) were used as beta-hemolysis controls. The presence or absence of zones of clearing around the colonies was interpreted as beta-hemolysis (positive hemolytic activity) or gamma-hemolysis (negative hemolytic activity), respectively. When observed, greenish zones around the colonies were interpreted as alpha-hemolysis and taken as negative for the assessment of hemolytic activity (Semedo *et al.*, 2003).

5.3.7.3. Virulence genes

Total DNA isolation was performed using a Gen Elute Bacterial Genomic DNA kit. The primers used for the amplification of genes *esp*, *agg*, *gel*E, *efaAfm* and *efaAfs*, *cylA*, *cylB* and *cylM* were described by Eaton and Gasson (2001) and primers of *cyl* operon: $cylL_L$ and $cylL_S$ were developed by Semedo *et al*. (2003). All the primers were purchased from MWG Biotech AG. PCR amplifications were performed in a ThermoCycler in 0.2 mL reaction tubes each with 25 µL of mixtures using 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.1 mM deoxynucleoside triphosphates (dNTP's), 0.5 mM of

each primer, 2 U of Taq DNA polymerase and 100 ng/μL of DNA. Amplification reactions were as follows: initial cycle of 94 ºC for 1 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. Amplification products were combined with 3 μL of loading buffer (Bio-Rad) and the preparation was electrophoresed on 0.8% (w/v) agarose gel at 90 V for 2 h. A 100-bp PCR DNA ladder was used as a molecular weight marker. The gels were photographed on an UV transilluminator and image analysis was accomplished using Quantity One software.

The positive controls used were: *Ent. faecalis* DS 16 ($cylL_L^+$, $cylL_S^+$), *E. faecalis* F2 ($cylA^+$, $cylB^+$, $cylM^+$, $efaAfs^+$), *Ent. faecium* F10 ($efaAfm^+$), *Ent. faecalis* P1 (agg^+, $gelE^+$) and *Ent. faecalis* P36 (esp^+) (from a collection of Tracy Eaton, Division of Food Safety Sciences, Institute of Food Research, Norwich, United Kingdom). For each PCR reaction a negative control (sample without template) was included.

5.3.7.4. Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC; μg/mL) of fourteen antibiotics was determined by the agar microdilution method, according to the National Committee for Clinical Laboratory Standards (NCCLS, 2004). The antibiotics investigated were vancomycin (Fluka), chloramphenicol (Fluka), nitrofurantoin (Sigma-Aldrich), erythromycin (Labesfal, Tondela, Portugal), tetracycline (Labesfal), ciprofloxacin (Labesfal), rifampicin (Labesfal), gentamicin (Labesfal), streptomycin (Sigma-Aldrich), oxacillin (Sigma-Aldrich), kanamycin (Fluka), ceftazidime (Sigma-Aldrich), penicillin G (Sigma-Aldrich) and ampicillin (Fluka). Antibiotic concentration ranged from 0.03 to 512 μg/mL.

MICs of trimethoprim/sulphamethoxazole (SXT, AB Biodisk, Solna, Sweden), meropenem (AB Biodisk) and imipenem (AB Biodisk), were determined using Etest method.

Each test was carried out on Muller-Hinton agar (MHA, BioMérieux) and on MHA with cations adjusted for penicillin G and ampicillin. The inoculum was prepared by suspending a culture in sterile Ringer's solution in order to obtain turbidity equivalent to 0.5 McFarland standards. The experiment was performed in duplicate. *Pediococcus pentosaceus* SB83 grown on plates of MHA and MHA with cations adjusted with no antibiotic was used as the negative control. *Enterococcus faecalis* ATCC 29212 and *E. coli* ATCC 259222 were used as quality control strains.

5.3.7.5. Biofilm assay

The quantification of biofilm production was performed as described previously by Borges *et al.* (2011). The wells of a sterile 96-well polystyrene microplate were filled with 180 µL of MRS broth or SVF, both media adjusted to pH values 4.2, 5.5 and 6.5 with HCl or NaOH; 20 µL of overnight grown cells suspended in Ringer´s solution was added to each well. The plates were incubated aerobically for 24 h, 48 h and 72 h at 37 ºC. To quantify the biofilm formation, the wells were gently washed three times with 250 µL of sterile-distilled water. The attached bacteria were fixed with 200 µL of methanol for 15 min and then microplates were emptied and dried at room temperature. Subsequently, 200 µL of a 2% (v/v) crystal violet solution was added to each well and held at ambient temperature for 5 min.

Excess stain was then removed by placing the plate under gently running tap water. Stain was released from adherent cells with 160 µL of 33% (v/v) glacial acetic acid. The OD of each well was measured at 630 nm using a plate reader. Each assay was done

eighteen times and the negative control was performed in uninoculated MRS broth or SVF. The cut-off (ODc) was defined as the mean OD value of the negative control. Based on the OD, strains were classified as: non-biofilm producers ($OD \leq OD_C$), weak ($OD_C < OD \leq 2 \times OD_C$), moderate ($2 \times OD_C < OD \leq 4 \times OD_C$) or strong biofilm producers ($4 \times OD_C < OD$).

5.3.8. Statistical analysis

An ANOVA was carried out to test the effect of SVF on survival of LAB strains and to test any significant effect of media, pH and time on biofilm formation of *P. pentosaceus* SB83. All calculations were carried out using the software Kaleidagraph.

5.4. Results

5.4.1. Screening for antimicrobial activity of lactic acid bacteria

Among the food isolates of LAB investigated, 62.5% (35/56) demonstrated anti-listerial activity, but did not inhibit GBS.

Clinical isolates of LAB did not show antimicrobial activity against *L. monocytogenes* or GBS.

Growth inhibition can result from competition, production of lactic acid, hydrogen peroxide or bacteriocins. According to the screening method used, all inhibitory isolates produced proteinaceous compounds that exhibited antimicrobial activity, (activity was lost only after the addition of trypsin). This result suggests the anti-listerial activity was caused by a bacteriocin.

5.4.2. Quantification of antibacterial activity

Bacteriocin activity of LAB isolates against *L. monocytogenes* isolates of three different serotypes varied between 400 to 6400 AU/mL, although values of 1600 and 3200 AU/mL (i.e. supernatants diluted 1600-fold and 3200-fold retained significant antilisterial activity) were more common.

One isolate of LAB (*P. pentosaceus* SB83) had a higher activity for all serotypes compared with the other LAB strains. This isolate showed an antibacterial activity of 6400 AU/mL for serotypes 1/2b and 4b and 3200 AU/mL for serotype 1/2a.

5.4.3. Identification of bacteriocin-producing lactic acid bacteria and RAPD-PCR

All strains with anti-listerial activity were identified as *Pediococcus* spp. by 16S rDNA sequencing; three isolates were identified as *P. acidilactici* and the remaining 32 isolates as *P. pentosaceus*.

A combined dendrogram for both primers M13 and D8635 was achieved and ten different profiles (clusters at 75%) were obtained for a cophenetic correlation value of 0.85 (data not shown). This value indicates a satisfactory representation of the similarity matrix in the dendrogram which should be very close to 1 for a high-quality solution.

5.4.4. Survival of lactic acid bacteria in simulated vaginal fluid

The SVF at normal pH (4.2) was used to determine the survival of *Pediococcus* spp. in these conditions.

In general, the behavior of strains in SVF was similar ($P = 0.31$) and the ten different profiles obtained using RAPD-PCR also had a similar behavior ($P = 0.38$).

The results demonstrated that in 24 h there were no great reductions in viable cells; (log (N/N_0) varied between 0.03 and -0.4), but at 48 h a greater reduction was observed. After 48 h, six isolates showed a decrease of more than 1.0 log. At this time, the most sensitive isolate had decreased by 1.6 log CFU/mL and the most resistant had reduced by only 0.2 log viable cells. Thus, after 48 h, survival was demonstrated to be strain dependent ($P < 0.001$).

Figure 5.1. presents the survival in SVF of *P. pentosaceus* SB83, at different time intervals (4, 8, 24 and 48 h).

All *Pediococcus* spp. isolates showed anti-listerial activity after 24 h of contact with SVF.

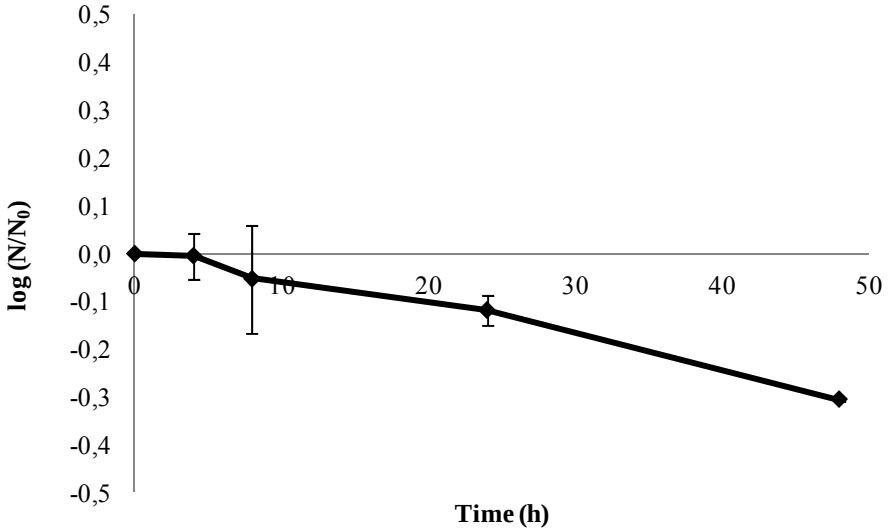

Figure 5.1. Survival of *P. pentosaceus* SB83 in SVF at pH = 4.2. All points are means ± standard deviations.

5.4.5. Characterization of *Pediococcus pentosaceus* SB83

5.4.5.1. Virulence factors (production of gelatinase, lipase and DNase, hemolytic activity and virulence genes)

The production of extracellular enzymes (gelatinase, lipase and DNase) and hemolytic activity were not demonstrated by *P. pentosaceus* SB83.

The presence of the surface adhesin genes (*efaAfs*, *efaAfm* and *esp*), the aggregation protein gene (*agg*), the cytolysin genes (*cylM*, *cylB*, *cylA*, $cylL_L$ $cylL_S$) and extracellular metallo-endopeptidase gene (*gelE*) were not present in *P. pentosaceus* SB83.

5.4.5.2. Antibiotic susceptibility testing

The antibiotic susceptibility of *P. pentosaceus* SB83 was evaluated for several antibiotics, in order to cover some of known chemical and functional classes of antibiotics, and MICs (μg/mL) were determined (Table 5.3.).

Table 5.3. Minimum Inhibitory Concentration (MIC; μg/mL) of seventeen antibiotics for *P. pentosaceus* SB83.

Antibiotic	Vancomycin	Chloramphenicol	Nitrofurantoin	Erythromycin	Tetracycline	Ciprofloxacin	Rifampicin	Gentamicin	Streptomycin	Oxacillin	Kanamycin	Ceftazidime	Penicillin G	Ampicillin	SXT	Meropenem	Imipenem
μg/mL	512	4	128	0.125	16	16	8	0.25	8	8	4	32	0.5	2	>32	1.5	0.125

5.4.5.3. Biofilm assay

Pediococcus pentosaceus SB83 produced biofilms in both medium (MRS and SVF) at different pH values (Figure 5.2.). In SVF, this strain was classified as a weak or moderate producer; in MRS, *P. pentosaceus* SB83 was classified as a moderate or strong producer. The quantity of biofilm produced was media dependent ($P < 0.001$).

In SVF, *P. pentosaceus* SB83 produced more biofilm at pH 4.2 (normal vaginal pH) than at higher pH values; at pH 5.5 and 6.5 biofilm production was not significantly different ($P = 0.13$). In MRS, biofilm production was pH dependent ($P < 0.001$); biofilm production increased with increasing pH.

At 24 h and 48 h, no significant differences were observed in production of biofilm at pH 5.5 and 6.5 (in both media). At pH 4.2, in SVF we did not observe significant differences over time ($P = 0.07$), whereas in MRS, biofilm formation was higher at 48 h, and no significant differences were observed after 24 h and 72 h incubation ($P = 0.28$).

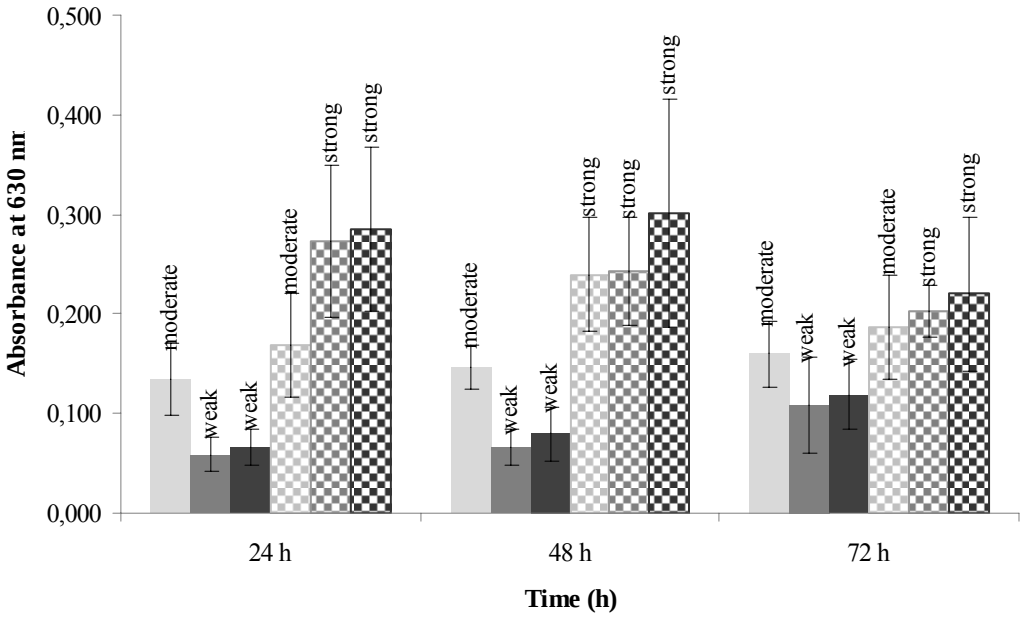

Figure 5.2. Production of biofilm by *P. pentosaceus* SB83 at different time intervals, in SVF and MRS broth at different values of pH. Optical density was measured in SVF at pH 4.2 (▫), 5.5 (▪) and 6.5 (■), and in MRS at pH 4.2 (▫), 5.5 (▪) and 6.5 (■) Based on the OD, the strain was classified as a strong, moderate or weak biofilm producer. Error bars represent standard deviation of the mean.

5.5. Discussion

Infection with *L. monocytogenes* can be acquired from animals through direct contact, from contaminated foods, via vertical transmission from mother to fetus/neonate through the placenta or birth canal, and via nosocomial cross-infection (Delgado, 2008, Posfay-Barbe and Wald, 2009). Listeriosis during pregnancy has many complications including pre-term labour, chorioamnionitis, spontaneous abortion, stillbirth and neonatal infection (DiMaio, 2000). Neonatal disease is classified as either early or late onset. Early onset is more likely in pre-term infants, probably resulting from *in utero* transmission. Late onset is more likely in term infants who acquired *L. monocytogenes* from the vaginal tract (or birth canal) of asymptomatic mothers. Listeriosis in the neonate is very similar to GBS infection in both course and treatment (Delgado, 2008).

Borges *et al.* (2011) demonstrated that *L. monocytogenes* can survive and proliferate in conditions of SVF, when women have an increased vaginal pH (pH of 5.5 and 6.5). An increase of pH is caused by a depletion of vaginal lactobacilli (Donders *et al.*, 2007, Simhan *et al.*, 2003), menstruation, unprotected sexual intercourse with the deposition of semen (Tevi-Bénissan *et al.*, 1997) and vaginal medications.

In this study, 35 isolates of LAB showed antimicrobial activity against clinical isolates of *L. monocytogenes*. Therefore, these isolates of LAB could be used to inhibit *L. monocytogenes*, preventing the previously mentioned sequelae caused by this pathogen. These LAB isolates were identified as *Pediococcu*s spp. and although they produce lactic acid, their antimicrobial activity was shown to be mainly caused by production of a bacteriocin. Several authors demonstrated that *L. monocytogenes* is highly resistant to acidic environments and strains of clinical origins are more resist to acid than isolates of food origin (Borges *et al.*, 2011; Ramalheira *et al.*, 2010).

Thereby, these isolates of *Pediococcus* spp. have two fields of action, on one side inhibit *L. monocytogenes* by action of a bacteriocin and on the other side maintain the acidic vaginal pH, which will prevent colonization and multiplication by other pathogenic microrganisms, including GBS.

Many other studies have demonstrated the bacteriocinogenic activity of *Pediococcus* spp. against isolates of *Listeria* spp. (Abrams *et al.*, 2011, Albano *et al.*, 2007, Anastasiadou *et al.*, 2008, Huang *et al.*, 2009, Pinto *et al.*, 2009).

Bacteriocin activity of *Pediococcus* spp. against *L. monocytogenes* of three different serotypes varied between 400 and 6400 AU/mL. Albano *et al.* 2007 reported two pediocins bacHA-6111-2 and bacHA-5692-3 with an activity of 1600 AU/mL against *L. innocua*.

Pediococcus spp. have been used as probiotics in foods (Jonganurakkun *et al.*, 2008; Ruiz-Moyano *et al.*, 2011), although may also be used in other fields. The anti-listerial *Pediococcus* spp. strains tested could potentially be used as probiotics for vaginal application, consequently it was important to evaluate their capacity to maintain their viability in a SVF. All isolates of *Pediococcus* spp. tested were able to survive in SVF at normal pH and all isolates demonstrated anti-listerial activity after exposure to SVF after 24 h. During 24 h, *Pediococcus* spp. maintained their survival and the greatest reduction was only 0.4 log CFU/mL. After 48 h only six isolates demonstrated a decrease of more than 1.0 log.

In our study, we used a SVF proposed by Owen and Katz (1999). This formulation has been approved for research into contraceptive and prophylactic drug delivery, thus some compounds present in vaginal fluid are not included (for example, not included are glycogen or mucin and the glucose concentration is low). However, even with the

exclusion of some ingredients *Pediococcus* spp. could survive, and were shown to be resistant isolates.

These results are in agreement with other reports which demonstrated the survival of *Lactobacillus* spp. in vaginal conditions (Geshnizgani and Onderdonk, 1992; Reid *et al.*, 1998; Tomás and Nader-Macías, 2007; Valore *et al.*, 2002).

The vaginal microbiota is normally dominated by lactobacilli, however many other LAB colonize women. In the study of Jin *et al.* (2007), a total of 338 vaginal lactic acid bacteria were isolated, including five genera: *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp. and *Weisella* spp.. The source of vaginal LAB has not been identified, but one source could be the environment, such as eating fermented food. In a study by Petricevic *et al.* (2012), 30 pregnant women and 30 post-menopausal women were investigated for LAB colonization; 80% of pregnant women and 40% of post-menopausal women had the same lactobacilli strains in their vagina and rectum. So these results support the hypothesis that the rectum may play an important role as a reservoir for some strains of LAB that colonise the vagina.

Therefore a bacterial replacement therapy controlling vaginal infections could be developed with isolates from food. Thus, the *Pediococcus* spp. used in this study could be used as a probiotic with vaginal application. Our isolates are able to inhibit the growth of *L. monocytogenes* by production of a bacteriocin and can survive in SVF. *Pediococcus pentosaceus* SB83 showed a greater bacteriocinogenic activity compared with other isolates. This strain maintained viability in SVF, showing only a slight decrease after 48 h exposure (0.3 cycle log).

Lactic acid bacteria have acquired the “generally regarded as safe” (GRAS) status by the American Food and Drug Administration. However, the safety of any strain of LAB should be evaluated before consideration for use as a vaginal probiotic.

Pediococcus pentosaceus SB83 did not produce virulence factors such as gelatinase, lipase and DNase, hemolytic activity and nor show the presence of virulence genes (genes of surface adhesion, aggregation protein, cytolysin and extracellular metallo-endopeptidase). Some of these virulence factors, frequently identified in pathogenic bacteria, have been detected in enterococci (Barbosa *et al.*, 2010).

The safety aspects of LAB are of concern, including the presence of potentially transferable antibiotic resistances to pathogenic bacteria (Ammor *et al.*, 2007). Probiotics can be susceptible to the majority of antibiotics or can naturally be, or rendered, multiresistant (Courvalin, 2006).

According to the microbiological breakpoints for antimicrobials defined by the Panel on Additives and Products or Substances in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA, 2008), *P. pentosaceus* SB83 was considered sensitive to cloramphenicol, gentamicin, streptomycin, kanamycin, erythromycin, and ampicillin. This strain was considered resistant to tetracycline and intrinsically resistant to vancomycin. The resistance to tetracycline was found in other studies of *Pediococcus* spp. (Danielsen *et al.*, 2007; Rojo-Bezares *et al.*, 2006; Swenson *et al.*, 1990; Tankovic *et al.*, 1993).

From the data obtained in the study by Klare *et al*. (2007), tentative species- or group-specific epidemiological cut-off (ECOFF) values of MICs were defined for recognizing intrinsic and acquired antimicrobial resistances. According to that study, the ECOFF for *P. pentosaceus* to oxytetracycline (class of tetracycline) was 32 µg/mL. Therefore, the tetracycline resistance of *P. pentosaceus* SB83 is likely to be intrinsic. Klare *et al.* (2007), did not observe an acquired antibiotic resistance in any isolates of the *Pediococcus* spp. tested.

Vancomycin resistance is due to their possession of D-Ala-D-Lactate in their peptidoglycan rather than the D-Ala-D-Ala dipeptide. Such resistance is thus intrinsic in most LAB because the antibiotic's target is absent and not comparable to the transmissible plasmid-encoded vancomycin resistance found in *Enterococcus* spp. Intrinsic resistance is not horizontally transferable, and poses no risk in non-pathogenic bacteria (Ammor *et al.*, 2007).

FEEDAP did not define the breakpoints for nitrofurantoin, ciprofloxacin, rifampicin, oxacillin, ceftazidime, penicillin, SXT, meropenem and imipenem.

In general, our results are in agreement with other studies for a broad range of antibiotics although different nutrient media, incubation conditions and susceptibility testing methods were used (Danielsen *et al.*, 2007; Klare *et al.*, 2007; Rojo-Bezares *et al.*, 2006; Swenson *et al.*, 1990; Tankovic *et al.*, 1993; Zaragaza *et al.*, 1999).

A biofilm is defined as a community of microorganisms attached to a surface, producing extracellular polymeric substances (EPS), exhibiting an altered phenotype compared with planktonic cells, especially with regard to gene transcription and interacting with each other (Lindsay and von Holy, 2006).

Pediococcus pentosaceus SB83 produced biofilm in MRS and SVF at different pH values. It was demonstrated that *P. pentosaceus* SB83 formed a greater quantity of biofilm in MRS than SVF fluid; medium composition influenced the quantity of biofilm production.

In MRS medium, biofilm production increased with increasing pH. In SVF, *P. pentosaceus* SB83 produced more biofilm at pH 4.2 (normal vaginal pH) than at higher pH values. Van der Veen and Abee (2011), studied the formation of single and mixed species biofilms of *L. monocytogenes* and *Lact. plantarum*, and the contribution of each species to the biofilm formation was dependent on the composition of medium. The

addition of glucose to the medium (Brain Heart Infusion) decreased the number of *L. monocytogenes* and increased the contribution of *Lact. plantarum* for the production of mixed biofilm. The acidification resulting from metabolism of glucose, allowed the formation of biofilm by *Lact. plantarum* because this strain is able to grown at low pH. The acid tolerance of LAB provides the opportunity to produced biofilm at acidic pH values; this capacity was evident in *P. pentosaceus* SB83 in the conditions of SVF at normal pH.

In a study by Guerrieri *et al.* (2009), LAB biofilms (of strains *Lact. plantarum* 35d, *Ent. casseliflavus* IM 416K1, *Lact. plantarum* 396/1, *Ent. faecalis* JH2-2) showed the ability to influence the survival and multiplication of *L. monocytogenes*. So the production of biofilm by *P. pentosaceus* SB83 in SVF can be an advantage in reduction of *L. monocytogenes.* Lactic acid bacteria biofilms also serve as a protective layer against the colonization by other pathogenic bacteria. These results should be complemented with adhesion assays to vaginal epithelial cells.

Cell adhesion and biofilm production play an important role in the stress resistance of LAB. Three mechanisms could increase the resistance of biofilm cells: the cell membrane becomes more resistant; the biofilm is protected by extracellular polymeric secretions, and the 3-dimensional structure of the biofilm protects the inner cells (Kubota *et al.*, 2008).

In conclusion, this study suggests that *P. pentosaceus* SB83 has the potential to be used as a vaginal probiotic, to prevent colonization of *L. monocytogenes* in pregnant women and consequently to reduce neonatal infections.

6. Characterization of a bacteriocin of *Pediococcus pentosaceus* SB83 and its potential for vaginal application

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6.1. Abstract

Listeria monocytogenes, the responsible agent for listeriosis, is one of several microorganisms which can be transmitted from mother to fetus/neonate by vertical transmission.

The aim of this study was to characterize a bacteriocin produced by *P. pentosaceus* SB83 for vaginal application, to prevent colonization of pregnant women with *L. monocytogenes*.

Bacteriocin SB83 showed inhibitory activity against *L. monocytogenes* (serotypes 1/2a 1/2b and 4b) but did not inhibit vaginal lactic acid bacteria. Additionally, bacteriocin SB83 inhibited the growth of *Ent. faecalis*, *Ent. faecium* and *Bacillus subtilis*.

Bacteriocin SB83 was produced at maximum levels between 24 h and 36 h of growth (6400 AU/mL against serotype 1/2b and 4b, 3200 AU/mL against serotype 1/2a). Significant reduction in antimicrobial activity was observed after treatment of cell-free supernatants with proteinase K, pepsin, papain and trypsin.

Bacteriocin SB83 was resistant to treatments with Tween 80, Tween 20, Triton X-100, SDS and EDTA. This bacteriocin was stable at pH values between 4 and 6.5; a decrease in activity was observed at pH 2 and above 8. A decrease in antibacterial activity was recorded at 100 ºC and 121 ºC. In the range 4 ºC to 80 ºC, antimicrobial activity was not affected. The activity of bacteriocin did not change in SVF (pH 4.2, 5.5 and 6.5).

The molecular size of bacteriocin SB83 was between 3.5 and 6.5 kDa.

In conclusion, the bacteriocin produced by *P. pentosaceus* SB83 is resistant to several conditions, including conditions in the vaginal tract (pH and components of vaginal fluid).

6.2. Introduction

Bacteriocins are ribosomally synthesized and are mainly known to have effective antimicrobial activity against pathogenic microorganisms (Ahmed *et al.*, 2010; Gillor *et al.*, 2005). They have a variety of action mechanisms, including cytoplasmic membrane pore formation, cell wall interference and nuclease activity (Gillor *et al.*, 2005).

Bacteriocins are categorized into different classes based on their biochemical and genetic properties. Class I bacteriocins are the lantibiotics, which are small and post-translationally modified to contain amino acids such as lanthionine and B-methyl-lanthionine, and several dehydrated amino acids. Class II includes unmodified heat-stable bacteriocins containing peptides with molecular masses of <10 kDa. Class II are subdivided into three subgroups, namely, class IIa (pediocin-like bacteriocins), class IIb (two-peptide bacteriocins), and IIc (other one-peptide bacteriocins). The class III peptides are thermosensitive proteins with molecular masses of >30 kDa (Drider *et al.*, 2006; Riley and Wertz, 2002). Another group, known as class IV, is often included in classification. Bacteriocins of class IV are complex molecules with lipid and carbohydrate moieties (Papagianni and Anastasiadou, 2009).

Bacteriocins can be used as antimicrobial agents for improving the safety and preservation of food products (Abrams *et al.*, 2011; Albano *et al.*, 2007) and for clinical therapeutics (Riaz *et al.*, 2010; Voravuthikunchai *et al.*, 2006). These proteinaceous molecules appear to be capable of displacing or suppressing the growth of other bacteria, and as such, perhaps provide an advantage to microorganisms in fermenting ecosystems, including the female genital tract (Boris and Barbés, 2000). As bacteriocins do not induce vaginal irritation they are suitable for human use (Dover *et al.*, 2008). Nisin, the lantibiotic produced by *Lactococcus lactis* strains, is a bacteriocin that is commercially available and well studied. Nisin received GRAS status by the Food and

Drug Administration (FDA) and it is the only bacteriocin that has been approved by the World Health Organization (WHO) for use for food preservation (Dover *et al.*, 2008; Mills *et al.*, 2011). Besides being used as food preservative, nisin is used as an antibacterial wipe for the udder prior to milking, it has also been suggested as a contraceptive agent (Dover *et al.*, 2008) and it has potential in treating peptic ulcer disease by inhibiting *Helicobacter pylori* growth and colonization (Gillor *et al.*, 2005). However, nisin may not be a good choice for vaginal application as it is strongly bactericidal for the healthy vaginal *Lactobacillus* spp. (Dover *et al.*, 2008).

Listeria monocytogenes, the responsible agent for listeriosis, is one of several microorganisms which can be transmitted from mother to fetus/neonate by vertical transmission, transplacentally or during passage through the birth canal. This infection is acquired because some women carry *L. monocytogenes* in the vaginal tract (Delgado, 2008; Posfay-Barbe and Wald, 2009). Vaginal infection during pregnancy represents many risks such as stillbirth, abortion, premature delivery or neonatal infection (Donati *et al.*, 2010).

Since some LAB exhibit antilisterial activity, these bacteria could be used in the prevention of neonatal listeriosis. Thus, the aim of this study was to characterize one bacteriocin produced by *P. pentosaceus* SB83 for potential vaginal application.

6.3. Materials and methods

6.3.1. Lactic acid bacteria

Pediococcus pentosaceus SB83 was selected because previous studies demonstrated that this strain is able to inhibit the growth of clinical isolates of *L. monocytogenes* (serotype 1/2a, 1/2b and 4b) by production of a bacteriocin. This strain has been shown to survive in SVF at normal vaginal pH (pH of 4.2) and *P. pentosaceus* SB83 does not

produce virulence factors such as gelatinase, lipase and DNase, hemolytic activity nor does it possess virulence genes (genes *esp*, *agg*, *gel*E, *efaAfm*, *efaAfs*, *cylA*, *cylB* and c*ylM*).

6.3.2. Bacteriocin activity spectrum

The antimicrobial activity of *P. pentosaceus* SB83 bacteriocin was tested against several Gram-negative and Gram-positive bacteria, according to the method described by Van Reenen *et al.* (1998). Lactic acid bacteria isolates were cultured in MRS broth at 37 ºC and all other isolates were grown in TSB at 37 ºC. Target bacteria were: 19 vaginal isolates of LAB, *E. coli* ATCC 259222, *Klebsiella pneumonia*e, *S. aureus* ATCC 25213, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Proteus vulgaris*, *Ent. gallinarium* DSMZ 20628, *Ent. gallinarium* DSMZ 20680, *Ent. flavescens* DSMZ 7370, *Ent. faecalis* DSMZ 12956, *Ent. faecium* DSMZ 13590, *Ent. faecalis* ATCC 2912, *Bacillus subtilis*, *Bacillus cereus*.

All strains were stored at -80 ºC in the respective growth media containing 30% (v/v) of glycerol.

6.3.3. Bacteriocin production during growth

MRS broth was inoculated with 1% (v/v) of *P. pentosaceus* SB83 culture (24 h at 37 ºC) and incubated at 37 ºC. Optical density (at 600 nm) and changes in pH of the culture were determined every hour for 48 h. Antimicrobial activity of the bacteriocin and viable counts were determined at 3 h intervals for 48 h. The enumeration of *P. pentosaceus* SB83 was performed by serial 10-fold dilutions in Ringer's solution and 20 µL were spread-plated in duplicate on MRS agar. Colony forming units per millilitre (CFU/mL) were determined after 48 h of incubation at 37 ºC. Antimicrobial activity

was expressed as AU/mL. AU is defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition of the target strains (Van Reenen *et al.*, 1998). *Listeria monocytogenes* strains of three different serotypes (1/2b (ESB 2076), 4b (ESB 2092) and 1/2a (ESB 2096)), deposited at *Listeria* culture collection of Escola Superior de Biotecnologia, were used as target strains.

6.3.4. Effect of various physical and chemical parameters on bacteriocin activity

Cell-free supernatant of LAB culture (24 h at 37 ºC), obtained by centrifugation (8877 x g, 10 min, 4 ºC), was adjusted to pH 6.0 with 1 M NaOH. Samples were used to evaluate the effect of enzymes, pH, detergents, temperature and components of vaginal fluid on bacteriocin activity. After each treatment, bacteriocin activity was determined as described above. Strains ESB 2076, ESB 2092 and ESB 2096 were used as target strains. The experiments were done in duplicate.

6.3.4.1. Effect of enzymes

Samples of 1 mL were incubated for 2 h at 37 ºC in the presence of 0.1 mg/mL and 1.0 mg/mL (final concentration) of trypsin, proteinase K (Bioron, Germany), pepsin (Sigma-Aldrich), papain (Sigma-Aldrich), lipase (Sigma-Aldrich) and catalase. All enzymes were dissolved in sodium phosphate buffer (pH 7.0), except pepsin which was dissolved in citric acid buffer (pH 3.0).

6.3.4.2. Effect of detergents

One milliliter of supernatant was incubated for 5 h at 37 ºC with 1% of Tween 20 (Sigma-Aldrich), Tween 80 (Sigma-Aldrich), Triton X-100 (Sigma-Aldrich), sodium dodecyl sulphate (SDS) (Sigma-Aldrich), or EDTA (0.1 mM, 2 mM and 5 mM) (Bio-Rad, Hercules, USA). Bacteriocin activity was also tested in SVF at pH 4.2, 5.5 and 6.5. The composition of vaginal fluid was described by Owen and Katz (1999): 3.5 g/L NaCl; 1.4 g/L KOH; 0.2 g/L $Ca(OH)_2$; 0.02 g/L bovine serum albumin; 2.0 g/L lactic acid; 1.0 g/L acetic acid; 0.2 g/L glycerol; 0.4 g/L urea; 5.0 g/L glucose (Owen and Katz, 1999).

6.3.4.3. Effect of pH

The effect of pH was performed by adjusting the pH of cell-free supernatant to a range values between pH 2.0 and 12.0 (at increments of two pH units) with 1 M HCl or NaOH. Bacteriocin activity was also tested at pH of 4.2, 5.5 and 6.5, as these pH values correspond to normal vaginal pH (pH 4.2) and also in the case of infection (pH 5.5 and 6.5) (Pavletic *et al.*, 2004). After 1 h of incubation at room temperature, the samples were readjusted to pH 6.0 and the activity was determined.

6.3.4.4. Effect of temperature

The effect of temperature on the antimicrobial activity of the bacteriocin was evaluated by incubating supernatant at 4, 25, 30, 37, 60, 80 and 100 ºC for 1 h and 2 h. Bacteriocin activity was also tested after 15 min at 121 ºC.

6.3.5. Mode of action

Twenty milliliters of the bacteriocin-containing cell-free supernatant (pH 6.0) was filter-sterilized and added to 100 mL early exponential phase (3-h-old) cultures of *L. monocytogenes* (ESB 2076, ESB 2092 and ESB 2096). Optical density at 600 nm was recorded every hour and viable cell counts of *L. monocytogenes* were determined at 3 h intervals for 12 h. The enumeration of *L. monocytogenes* was performed by serial 10-fold dilutions in Ringer's solution and samples were plated in duplicate on TSA-YE. CFU per milliliter were also determined after 24 h of incubation at 37 ºC. *Listeria monocytogenes* cultures without added bacteriocin were used as controls.

6.3.6. Adsorption of the bacteriocin to the producer cells

Adsorption of bacteriocin to producer cells was performed according to Yang *et al.* (1992). After incubation for 24 h at 37 ºC, the culture of *P. pentosaceus* SB83 in MRS broth was adjusted to 6.0 with 1 M NaOH to allow maximal adsorption. The cells were harvested by centrifugation (8877 x g, 15 min, 4 ºC) and washed with sterile 0.1 M phosphate buffer (pH 6.5). The cells were re-suspended in 10 mL 100 mM NaCl (pH 2.0) and agitated for 1 h at 4 ºC. Subsequently, cells were harvested (8877 x g, 15 min, 4 ºC), the supernatant was neutralized to pH 6.0 and tested for bacteriocin activity (AU/mL).

6.3.7. Determination of approximate molecular size of bacteriocin by SDS-PAGE

Pediococcus pentosaceus SB 83 was grown in MRS broth for 24 h at 37 ºC, cells were harvested (8877 x g, 15 min, 4º C) and ammonium sulphate gradually added to the supernatant to 40% saturation. After 4 h of slow stirring at 4 ºC, the proteins were

harvested (8877 x g, 20 min, 4 ºC). Precipitated proteins were resuspended in one tenth-volume 25 mM ammonium acetate buffer (pH 6.5) and then separated by tricine-SDS-PAGE, as described by Schägger and Von Jagow (1987). A low molecular weight marker with sizes ranging from 1.060 to 26.600 kDa (Sigma-Aldrich) was used. The gels were fixed and one half was stained with Coomassie Brilliant Blue R250 (Bio-Rad). The position of the active bacteriocin was determined by overlaying the other half of the gel (not stained and washed with sterile distilled water) with cells of *L. monocytogenes* embedded in TSB-YE + 1% (w/v) agar.

6.4. Results

6.4.1. Bacteriocin activity spectrum

Besides the antimicrobial activity against *L. monocytogenes*, bacteriocin produced by *P. pentosaceus* SB83 also demonstrated inhibition of *Ent. faecium* DSMZ 13590, *Ent. faecalis* ATCC 2912, *Ent. faecalis* DSMZ 12956 and *Bacillus subtilis*. It should be noted that this bacteriocin had no activity against all isolates of vaginal LAB tested.

6.4.2. Bacteriocin production during growth

Bacteriocin production reached maximum levels against the three serotypes of *L. monocytogenes* between 24 h and 36 h of growth (Figure 6.1). There was a large decrease of pH during the first 24 h of growth (pH decreased to 4.0), then the pH was maintained finally declining to pH 3.8 after 48 h of incubation (Figure 6.1). During the 48 h of incubation, the viable cell count of *P. pentosaceus* SB83 increased approximately 3.6 log CFU/mL (data not shown).

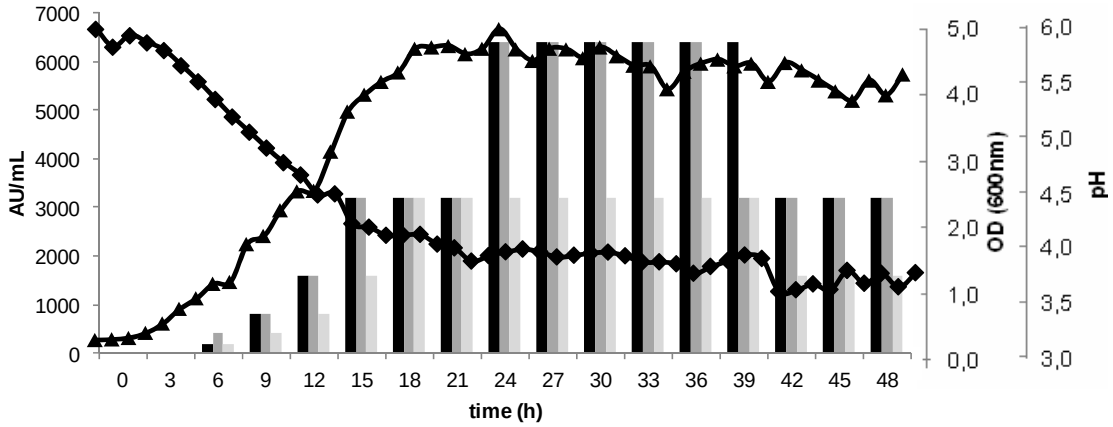

Figure 6.1. Production of bacteriocin by *P. pentosaceus* SB83. Antimicrobial activity is presented as AU/mL (bars), against *L. monocytogenes* ESB 2076 (■), *L. monocytogenes* ESB 2092 (■) and *L. monocytogenes* ESB 2096 (■). Changes in optical density (▲) and pH values are indicated (♦).

6.4.3. Effect of various physical and chemical treatments on bacteriocin activity

The activity of bacteriocin was not greatly affected by temperature; there was only a significant reduction during exposure to high temperatures (100 and 121 ºC). At lower temperatures (4 and 25 ºC), there was a decrease of activity to serotype 4b (14%) only.

In relation to pH, bacteriocin remained stable within the range of pH values of 4 and 6.5. At pH 2, 8 and 10 a decrease in activity was observed, though at pH 12 the reduction was more marked. At this pH there was a reduction of approximately of 50% for serotypes 1/2b and 4b, while for serotype 1/2a total loss of the activity of the bacteriocin was recorded.

Bacteriocin activity was reduced during incubation with all proteolytic enzymes; activity was completely lost after treatment with proteinase K and pepsin (0.1 and 1.0

mg/mL), but no change in activity was recorded when treated with catalase and lipase (0.1 and 1.0 mg/mL).

Bacteriocin SB83 was resistant to treatment with different detergents. Only EDTA decreased the activity against serotype 1/2b (14%).

The activity of the bacteriocin did not change in SVF at pH 4.2, 5.5 and 6.5 (Table 6.1.).

Table 6.1. Reduction of bacteriocin activity after incubation in different conditions (expressed in percentage values).

	Listeria monocytogenes	ESB 2076 (1/2b)	ESB 2092 (4b)	ESB 2096 (1/2a)
Temperature	**4 ºC**	0	14	0
	25 ºC	0	14	0
	30 ºC	0	0	0
	37 ºC	0	0	0
	60 ºC	0	0	0
	80 ºC	0	0	0
	100 ºC	14	14	17
	121 ºC	43	29	17
pH	**2.0**	14	14	17
	4.0	0	0	0
	4.2	0	0	0
	5.5	0	0	0
	6.0	0	0	0
	6.5	0	0	0
	8.0	14	14	17
	10.0	29	14	17
	12.0	57	43	100
Enzymes	**Proteinase K** $_{0.1 \text{ and } 1.0 \text{ mg/mL}}$	100	100	100
	Papain $_{0.1 \text{ mg/mL}}$	29	29	67
	Papain $_{1.0 \text{ mg/mL}}$	57	57	83
	Pepsin $_{0.1 \text{ and } 1.0 \text{ mg/mL}}$	100	100	100
	Trypsin $_{0.1 \text{ mg/mL}}$	29	43	33
	Trypsin $_{1 \text{ mg/mL}}$	57	57	50
	Catalase $_{0.1 \text{ and } 1.0 \text{ mg/mL}}$	0	0	0
	Lipase $_{0.1 \text{ and } 1.0 \text{mg/mL}}$	0	0	0
Detergents	**Tween 20**	0	0	0
	Tween 80	0	0	0
	Triton X-100	0	0	0
	SDS	0	0	0
	EDTA 0.1mM	14	0	0
	EDTA 2.0 mM	14	0	0
	EDTA 5.0 mM	14	0	0
Vaginal fluid	**pH 4.2**	0	0	0
	pH 5.5	0	0	0
	pH 6.5	0	0	0

6.4.4. Mode of action (determination of the reduction of viable cells of test microorganisms in the presence of bacteriocin)

Addition of bacteriocin SB83 to a 3-h-old culture of *L. monocytogenes* (early exponential phase) suppressed the growth for at least 9 h (Figure 6.2.). After addition of bacteriocin a decrease in viable cell numbers of *L. monocytogenes* ESB 2076 (1/2b), ESB 2092 (4b) and ESB 2096 (1/2a) (approximately 4 log CFU/mL within 3 h) was observed. However, prolonged incubation resulted in increase in CFU (data not shown). After 9 h of exposure, there is an increase of 0.7 log for serotype 1/2b, 1.1 log for serotype 4b and 1.0 log for serotype 1/2a.

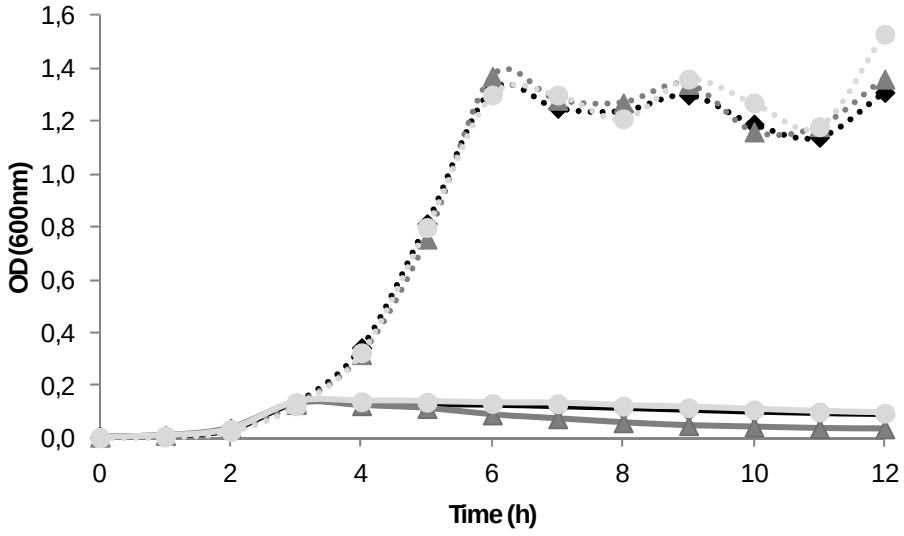

Figure 6.2. Effect of bacteriocin SB83 on *L. monocytogenes* ESB 2076 (1/2b) (♦), *L. monocytogenes* ESB 2092 (4b) (▲) and *L. monocytogenes* ESB 2096 (1/2a) (●). The solid line indicates the effect of bacteriocin on *L. monocytogenes* and the dotted line represents the growth of *L. monocytogenes* without added bacteriocin (controls).

6.4.5. Adsorption of the bacteriocin to the producer cells

After treatment of cells of *P. pentosaceus* SB83 with 100 mM NaCl (pH 2.0), the cellular supernatant did not show antimicrobial activity, suggesting that the bacteriocin did not adhere to the surface of the producer cells.

6.4.6. Determination of approximate molecular size of bacteriocin by SDS-PAGE

The molecular weight of the bacteriocin produced by *P. pentosaceus* SB83, is between 3.5 and 6.5 kDa (Figure 6.3.).

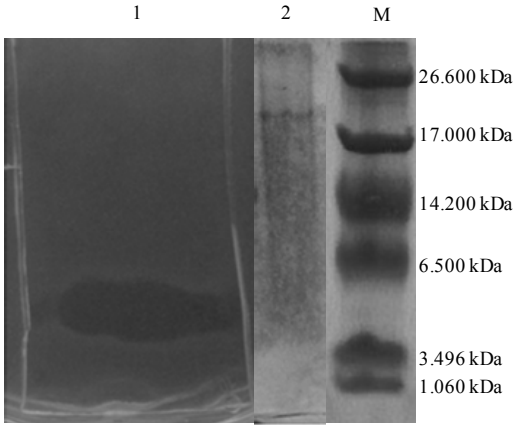

Figure 6.3. Tricine-SDS-PAGE of bacteriocin SB83. Lane 1: the gel overlaid with the indicator strain and respective zone of the growth inhibition; lane 2: peptide band stained with Coomassie Blue R250 (40% ammonium sulphate saturated); lane M: molecular weight marker.

6.5. Discussion

Bacteriocins have been considered promising natural entities for application in food preservation or medical treatment due to their effective antimicrobial activity against several pathogens, and their metabolic properties.

In the present study, a bacteriocin, produced by *P. pentosaceus* SB83, was characterized. This bacteriocin inhibits *L. monocytogenes* but not vaginal LAB.

An extensive number of studies report the bacteriocinogenic activity of *Pediococcus* spp. against food isolates of *Listeria* spp. (Abrams *et al.*, 2011; Albano *et al.*, 2007; Anastasiadou *et al.*, 2008; Huang *et al.* 2009; Pinto *et al.*, 2009).

Some studies report the bacteriocinogenic activity of vaginal isolates, however, they inhibit both pathogens and also healthy vaginal microbiota (Dezwaan *et al.*, 2007; Karaoğlu *et al.*, 2002).

Bacteriocin SB83 also inhibited the growth of *Ent. faecalis*, *Ent. faecium* and *B. subtilis*. The spectrum of antimicrobial activity against Gram-positive bacteria is characteristic of many class IIa bacteriocin. No antimicrobial activity was observed against Gram-negative bacteria. The structure and composition of the outer membrane of Gram-negative bacteria does not allow access of pediocin to the cytoplasmic membrane. Only a few strains of *P. pentosaceus* were described as being active against Gram-negative bacteria (Spelhaug and Harlander, 1989). The inhibition of *Enterococcus* spp. would be interesting in the prevention of urinary tract infections.

Bacteriocin SB83 was produced at maximum levels against three serotypes *of L. monocytogenes* (1/2a, 1/2b and 4b) between 24 h and 36 h. There was a large decrease of pH during the growth of *P. pentosaceus* SB83. This result was similar to other studies (Aroutcheva *et al.* 2001; Juarez Tomás *et al.*, 2002). The bacteriocin SB83 was resistant to temperature. Kelly *et al*. (2003) studied a vaginal enterocin that was heat and cold stable. In their study, the inhibitor appeared heat stable following exposure to 50, 60, 80 or 100 ºC, but lost activity at 121 ºC. At 4 ºC the enterocin also remained stable. Most pediocin and pediocin-like bacteriocins produced by *Pediococcus* spp. are

thermostable (Abrams *et al.*, 2011; Albano *et al.*, 2007; Huang *et al.*, 2009; Todorov and Dicks, 2009).

Bacteriocin SB83 was stable at pH values between 4 and 6.5. Since the context of the current study was the production of antimicrobial substance by *P. pentosaceus* SB83 to use in the vaginal tract, it was notable that bacteriocin was stable over pH range associated with a healthy vaginal environment (4-4.2) and in vaginal infection (5.5 and 6.5). Bacteriocins of vaginal isolates have been shown to be stable at acidic pH (Dezwaan *et al.*, 2007; Karaoğlu *et al.*, 2002).

No change in activity was observed when treated with catalase, indicating that H_2O_2 was not responsible for inhibition. Antimicrobial activity was decreased with papain and typsin, and was completely lost with proteinase K and pepsin. Studies performed with vaginal bacteriocins also showed complete loss of antimicrobial activity with addition of proteinase K (Karaoğlu *et al.*, 2002) and pepsin (Dezwaan *et al.*, 2007). Bacteriocins produced by different *Pediococcus* spp. show a slight resistance to proteolytic enzymes (Abrams *et al.*, 2011; Anastasiadou *et al.*, 2008). Treatment with lipase did not modify the antimicrobial activity, suggesting that lipids are not involved in the structure of the active molecule or molecular complex.

Bacteriocin SB83 was resistant to different detergents tested, Tween 80, Tween 20, Triton X-100, SDS and EDTA. Similar results were reported for a bacteriocin produced by others strains of *P. pentosaceus* (Abrams *et al.*, 2011; Pinto *et al.*, 2009; Todorov and Dicks, 2009).

A number of factors must be considered when choosing a bacteriocinogenic strain for vaginal application. Therefore, we considered it was relevant to assess the influence of vaginal fluid on the activity of bacteriocin. In the present study, the bacteriocinogenic activity did not change in SVF at pH 4.2, 5.5 and 6.5.

The addition of bacteriocin to a 3-h-old culture of *L. monocytogenes* (early exponential phase) suppressed the growth for at least 9 h. Similar results were recorded for other bacteriocins produced by *Pediococcus* spp. against *Listeria* spp. (Abrams *et al.*, 2011; Albano *et al.*, 2007). In the study of Todorov *et al*. (2007), a *Lactococcus lactis* spp. lactis HV219, isolated from vaginal secretion produced a bacteriocin that inhibited *Lactobacillus* spp. and *Enterococcus* spp. The treatment of early exponential phase cells *of Ent. faecium* and *Ent. faecalis* with the bacteriocin HV219 resulted also in an immediate and complete growth inhibition for 10 h.

In our study, the addition of bacteriocin to *L. monocytogenes* (1/2a, 1/2b and 4b) caused a drastic decrease in viable cells (approximately 4 log in 3 h), that suggested the mode of action of bacteriocin was bactericidal. However, prolonged incubation (9 h) resulted in a slight increase in CFU/mL which may be associated with an insufficient concentration of the bacteriocin or degradation by proteases (Huang *et al.*, 2009; Kingcha *et al.*, 2012).

Bacteriocin SB83 did not adhere to the surface of the producer cells. Other studies performed with *Pediococcus* spp. demonstrated the same results (Abrams *et al.*, 2011; Albano *et al.*, 2007; Huang *et al.*, 2009). The autoimmunity of producer cells probably resulted from either absence or masking of cell receptors for adsorption (Barefoot and Klaenhammer, 1983).

The molecular size of bacteriocin SB83 was between 3.5 and 6.5 kDa, as determined by tricine-SDS-PAGE. Most bacteriocins (pediocin and pediocin-like) produced by *Pediococcus* spp. have antilisterial activity, are thermostable and are low molecular weight (<10 kDa) (Abrams *et al.*, 2011; Albano *et al.*, 2007; Todorov and Dicks, 2009).

The effectiveness of the action of *Pediococcus* spp. with antilisterial activity has been tested in various foods, particularly meat and dairy products. However, according to our

knowledge this is the first study with *Pediococcus* spp. for vaginal application. *Pediococcus pentosaceus* SB83 produces a bacteriocin that is stable in conditions of vaginal fluid (components and pH), that is resistant to different detergents and does not inhibit the vaginal lactobacilli. These characteristics are important for application of this bacteriocin in the vaginal tract.

Natural antimicrobials, while inhibiting growth of pathogens, will help the survival and promote the growth of the native *Lactobacillus* spp., thus supporting the natural defenses against pathogenic organisms. Further research on the technological properties of *P. pentosaceus* SB83 must be done to determine its potential as a vaginal probiotic.

7. Effects of processing and storage on *Pediococcus pentosaceus* SB83 in vaginal formulations: lyophilized powder and tablets

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7.1. Abstract

Vaginal probiotics have an important role in preventing the colonization of the vagina by pathogens. This study aimed to investigate different formulations with *P. pentosaceus* SB83 (lyophilized powder and tablets with and without retarding polymer) in order to verify its stability and anti-listerial activity after manufacture and during storage. The bacteriocinogenic activity of *P. pentosaceus* SB83 against *L. monocytogenes* was evaluated in simulated vaginal fluid. Suspension of *P. pentosaceus* SB83 reduced the pathogen only after 2 h, the lyophilized bacteria after 24 h of contact and in the tablets, *P. pentosaceus* SB83 lost the antimicrobial activity. The pH of SVF decreased for all the tested conditions.

As lyophilized powder demonstrated better results concerning antimicrobial activity, this formulation was selected to evaluate the anti-listerial activity during the 12 months of storage. During storage at room temperature, lyophilized bacteria totally inhibited the pathogen only until one month of storage. At 4 ºC, *P. pentosaceus* SB83 showed antimicrobial activity during all the time of storage investigated.

Therefore, the better formulation of *P. pentosaceus* SB83 is the lyophilized powder stored at 4 ºC, which may be administered intra-vaginally as a washing solution.

7.2. Introduction

The dominant microorganisms in vaginal microbiota are lactobacilli, with a concentration of 10^7 to 10^8 CFU/mL of vaginal fluid in healthy premenopausal women (Boris and Barbés, 2000; Farage *et al.*, 2010). However, other LAB genera have been found in the vaginal tract, such as *Pediococcus* spp., *Weisella* spp., *Streptococcus* spp. and *Leuconostoc* spp. (Jin *et al.*, 2007). Moreover, it is acknowledged that the composition of the microbiota can vary from day to day, even in women without an indication of infections (Schwebke, 2001).

The normal vaginal pH of healthy woman is acidic (4-4.5), with variations from 6.6 (+/- 0.3) to 4.2 (+/- 0.2) between day 2 and day 14 of the menstrual cycle (Charlier *et al.*, 2009), although vaginal pH can rise with the diminishing of LAB existing in the vaginal microbiota, since glucose is not converted to lactic acid. High pH values promote growth of pathogenic organisms, particularly colonization by enteric bacteria (Farage *et al.*, 2010).

The microorganisms that are normally present in the vaginal environment, play a major role in preventing illnesses of the host, including BV, UTI, yeast vaginitis, and sexually transmitted diseases including HIV (Reid and Bocking, 2003). The vaginal LAB are also able to reduce the incidence of ascending infections of the uterus and the subsequent production of pro-inflammatory molecules (Wilks *et al.*, 2004). During pregnancy, several genital microorganisms such as *E. coli*, *L. monocytogenes* and viridans streptococci may be involved in chorioamnionitis (Donati *et al.*, 2010).

Antimicrobial treatment of urogenital infections is not always effective, and problems remain due to bacterial and yeast resistance. To minimize recurrent infections, as well as side effects, it is important develop and produce alternative treatments or drugs (Cribby *et al.*, 2008).

Due to the beneficial characteristics of LAB, there has been a growing interest in the potential use of LAB as probiotics for maintaining normal urogenital health (Anukam, 2007). The World Health Organization and the Food and Agriculture Organization of the United Nations have defined probiotics as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host" (Iannitti and Palmieri, 2010).

A probiotic may act indirectly through treating and preventing recurrent BV or directly by secreting substances (e.g. hydrogen peroxide, bacteriocins, lactic acid) that inhibit pathogens (Bolton *et al.*, 2008).

Vaginal dosage forms available include creams, gels, tablets, capsules, pessaries, foams, ointments, films, tampons, rings, and douches. While the majority of vaginal drugs so far have been in the form of gels, there is a growing interest in alternative dosage forms such as rings, tablets, and films. The majority of issues with product development and scale-up are similar to other pharmaceutical products, although there are some unique challenges because of the site of delivery, prophylactic nature of product application, and diversity in sex and hygiene practices across the developing world (Garg *et al.*, 2010).

The purpose of this study was to develop a vaginal formulation containing viable cells of a LAB isolate which can be used by women to prevent vaginal colonization of microorganisms (for example, *L. monocytogenes*) that may be harmful to the fetus/neonate. In this study the probiotic properties (survival and antagonistic activity) of formulations were evaluated after manufacture and during long-term storage of the product at different temperatures.

7.3. Material and methods

7.3.1. Microorganisms and culture conditions

Pediococcus pentosaceus SB83 (Escola Superior de Biotecnologia Culture Collection) was selected because previous studies demonstrated that this strain is able to inhibit the growth of clinical isolates of *L. monocytogenes* (serotype 1/2a, 1/2b and 4b) by production of a bacteriocin. This strain has been shown to survive in SVF at normal vaginal pH (pH of 4.2) and *P. pentosaceus* SB83 does not produce virulence factors such as gelatinase, lipase and DNase, hemolytic activity, nor does it possess virulence genes (genes *esp*, *agg*, *gel*E, *efaAfm*, *efaAfs*, *cylA*, *cylB* and c*ylM*) (Borges *et al.*, 2013b).

Pediococcus pentosaceus SB83 was grown on MRS agar and was sub-cultured twice in MRS broth at 37 ºC for 24 h before use in all tests.

The pathogenic bacteria, *L. monocytogenes* 2092 4b (isolated from blood; Escola Superior de Biotecnologia Culture Collection), was grown on TSA-YE and sub-cultured in TSB-YE at 37 ºC for 24 h.

All strains were stored at -80 ºC in the presence of 30% (v/v) glycerol.

7.3.2. Preparation of freeze-dried cells

After growth of *P. pentosaceus* SB83 in MRS broth, cultures were harvested by centrifugation at 8877 x g for 10 min at 4 ºC and washed twice in sterile Ringer's solution. The harvested cells were then suspended to the original volume in 11% (w/v) of skim milk (Oxoid). The cultures were frozen at -80 ºC for 24 h and subsequently freeze-dried (Martin Christ, Osteradam Harz, Germany) for 2-3 days.

7.3.3. Preparation of vaginal tablets

The formulations of vaginal tablets constituted different excipients: 3.3% talc (Acofarma, Barcelona, Spain), 3.0% magnesium stearate (J.M.V. Pereira, Portugal), 0.2% colloidal silicon dioxide (Acofarma), 7.5% hydroxypropylmethylcelluloses (HPMC, Acofarma) as retarding polymer and 86.0% of lyophilized bacteria. Simultaneously, tablets without the retarding polymer were also prepared.

The HPMC are known to show bioadhesive properties, but they are also able to hydrate and gel very slowly, due to their high molecular weights and viscosities, for this reason, the tablet dissolves over a longer time and provides a prolonged release of the active ingredient embedded in the polymeric matrix (Maggi *et al.*, 2000). The powder contained an amount of magnesium stearate as lubricant and talc as anti-adherent (Klayraung *et al.*, 2009).

All compounds were mixed in a Turbula apparatus for 15 min. Then, tablets were prepared by direct compression using a single-punch tablet press (Specac Ltd., Kent, United Kingdom). A quantity of the mixture (approximately 600 mg) was filled into a die of 11 mm diameter and under a pressure of 9.8 kN, tablets with a plane surface were formed.

The possible adverse effects of pressing on bacterial viability were investigated by comparing the viability of cells in tablets with those in formulated powders.

7.3.4. Release study of vaginal tablets

The determination of the release of *P. pentosaceus* SB83 from vaginal tablets was done using SVF. Simulated vaginal fluid was prepared according to Owen and Katz (1999): 3.5 g/L NaCl; 1.4 g/L KOH; 0.2 g/L $Ca(OH)_2$; 0.02 g/L bovine serum albumin; 2.0 g/L lactic acid; 1.0 g/L acetic acid; 0.2 g/L glycerol; 0.4 g/L urea; 5.0 g/L glucose. Once

these compounds were combined in solution, the mixture was adjusted to a pH of 4.2, 5.5 and 6.5 using HCl or NaOH. These pH values were used to reproduce the vaginal pH in normal conditions (pH 4.2) and in the case of infection (pH 5.5 and 6.5). As control, Ringer's solution was used.

Tablets with and without retarding polymer were placed in ten millilitres of each solution at 37 ºC, since daily production of vaginal fluid ranges between 1.89-11.00 mL/day (Owen and Katz, 1999). At appropriate time intervals, aliquots were withdrawn for further enumeration of viable bacteria. Two independent replicates of these assays were performed. The enumeration of *P. pentosaceus* SB83 was performed by serial 10-fold dilutions in Ringer's solution and 20 µL were spread-plated in duplicate on MRS agar. Colony forming units per gram (CFU/g) were determined after 48 h of incubation at 37 ºC.

7.3.5. Viability of *Pediococcus pentosaceus* SB83 in tablets and lyophilized powder

Freeze-dried powder and pharmaceutical preparations (freeze-dried powder with excipients and tablets) were stored in plastic containers at room temperature and at 4 ºC for 12 months. At several time intervals (0, 1, 3, 6, 9 and 12 months), the viability of *P. pentosaceus* SB83 was determined by plate method using MRS agar medium (described above). The detection limit of the enumeration technique was 1.4 log CFU/g.

7.3.6. Evaluation of antimicrobial activity of *Pediococcus pentosaceus* SB83 in simulated vaginal fluid

Pediococcus pentosaceus SB83, produce a bacteriocin against *L. monocytogenes* that is resistant to components and pH of vaginal fluid (Borges *et al.*, 2013a).

The bacterial suspension, lyophilized powder and tablets were used in these experiments to evaluate the antimicrobial activity of *P. pentosaceus* SB83 in SVF. To obtain the bacterial suspension, the microorganism was sub-cultured twice (24 h at 37 ºC) in 10 mL MRS broth, using a 1% v/v inoculum. Then cultures were harvested by centrifugation at 8877 x g for 10 min at 4 ºC, washed twice in sterile Ringer's solution and the pellet of bacteria was used. The lyophilized powder and tablets were prepared as described above.

Simulated vaginal fluid at pH of 6.5 was inoculated with an overnight culture of *L. monocytogenes* 2092 4b suspended in Ringer's solution with a final concentration of 10^5 CFU/mL. Then, *P. pentosaceus* SB83 (bacterial suspension, lyophilized powder or tablet) was added.

During 24 h, aliquots were withdrawn for further enumeration (*P. pentosaceus* SB83 and *L. monocytogenes* 2092 4b) and for pH measurement. The enumeration of *P. pentosaceus* SB83 was performed on MRS agar and the enumeration of *L. monocytogenes* on PALCAM agar (Merck), after 48 h incubation at 37 ºC.

The experimental conditions were: (1) SVF inoculated with *P. pentosaceus* SB83 (bacterial suspension, lyophilized powder, tablet with retarding polymer and tablet without retarding polymer) and *L. monocytogenes* 2092 4b, (2) SVF with 1 mg/mL of trypsin and inoculated with *P. pentosaceus* SB83 and *L. monocytogenes* 2092 4b, (3) SVF inoculated with a non-bacteriocinogenic LAB strain ESB67 (Escola Superior de Biotecnologia Culture Collection) and *L. monocytogenes* 2092 4b, (4) SVF inoculated with *P. pentosaceus* SB83, (5) SVF inoculated with *L. monocytogenes* 2092 4b. Each trial was performed in duplicate.

After this experiment, the formulation of *P. pentosaceus* SB83 that demonstrated the better results of anti-listerial activity was selected; the formulation was then stored at

room temperature and 4 ºC for 12 months. At 1, 3, 6, 9 and 12 months, the evaluation of antimicrobial activity of *P. pentosaceus* SB83 was performed as previously described.

7.3.7. Statistical analysis

An ANOVA was carried out to assess: (1) the effect of pH in release of *P. pentosaceus* SB83 in tablets with and without retarding polymer, (2) the effect of time and temperature of storage on viability of *P. pentosaceus* SB83 in tablets and lyophilized powder, (3) the effect of different forms of *P. pentosaceus* SB83 (bacterial suspension, lyophilized powder and tablets) for anti-listerial activity and reduction of pH of SVF, (4) the effect of storage on anti-listerial activity and reduction of pH of SVF. All calculations were carried out using the software Kaleidagraph.

7.4. Results

7.4.1. Enumeration of *Pediococcus pentosaceus* SB83 in lyophilized powder and tablets

The number of *P. pentosaceus* SB83 in lyophilized powder and in tablets (with and without retarding polymer) was 10^{10} CFU/g.

7.4.2. Release study of vaginal tablets

The release of *P. pentosaceus* SB83 from tablets with and without retarding polymer is shown in Figure 7.1.

In tablets with retarding polymer, *P. pentosaceus* SB83 was not completely released in all media tested. In SVF at pH 4.2 a lower release was observed (approximately 5.0 log CFU/g), than the release in the other media (SVF at pH 5.5, SVF at pH 6.5 and Ringer's solution) that was approximately 8.0 log CFU/g.

In tablets without retarding polymer, *P. pentosaceus* SB83 was completely released after 48h, in SVF at pH 5.5, SVF at pH 6.5 and Ringer's solution. The release of bacteria was lower in SVF at pH 4.2, values being approximately 8.0 log CFU/g.

Therefore, in both tablets (with and without retarding polymer) the bacteria release characteristics were found to be different when comparing the release in SVF at pH4.2 and the other media ($P < 0.0001$).

In either of the tablets, there was a large release of bacteria in the early hours of contact.

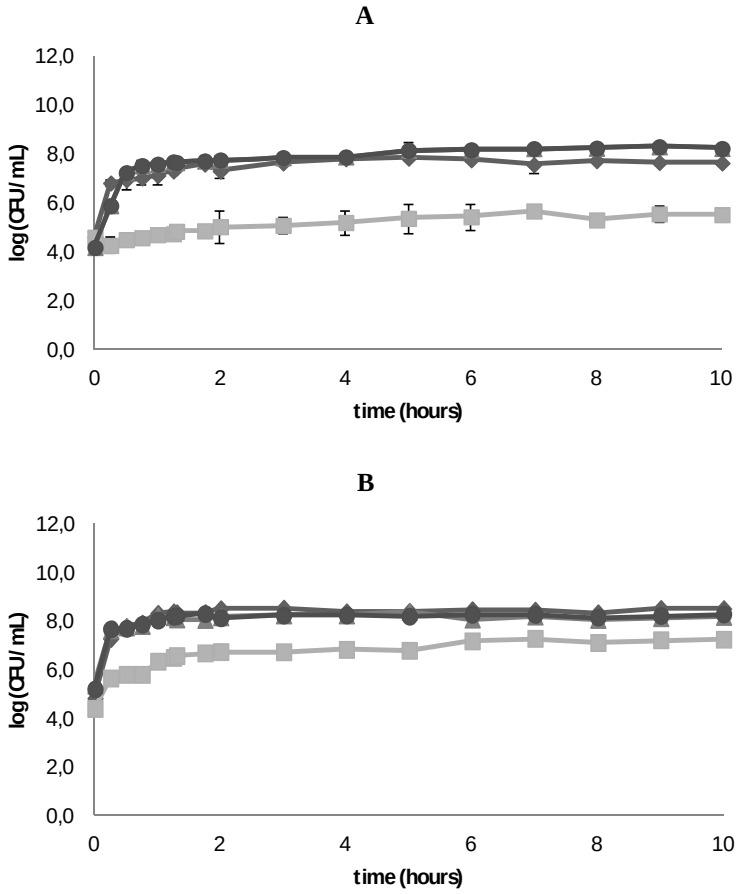

Figure 7.1. Release of *P. pentosaceus* SB83 from (A) tablets with retarding polymer and (B) tablets without retarding polymer. The release of bacteria was tested in SVF at pH = 4.2 (■); pH = 5.5 (▲); pH = 6.5 (●) and in Ringer's solution (♦). All points are means ± standard deviations.

7.4.3. Viability of *Pediococcus pentosaceus* SB83 in tablets and lyophilized powder

Figure 7.2 shows the viability of *P. pentosaceus* SB83 in lyophilized powder and pharmaceutical formulations (lyophilized powder with excipients and tablets) during storage at room temperature and 4 ºC.

At room temperature, in conditions with retarding polymer (Figure 7.2., graph A), the survival was similar during 9 months storage ($P = 0.09$). However, after 12 months of storage, the tablets showed a greater decrease of viable cells (reduction of 9.5 log CFU/g) than lyophilized powder and lyophilized powder with excipients. In conditions without retarding polymer (Figure 7.2., graph B), no significant differences were found between lyophilized, lyophilized with excipients and tablets ($P = 0.18$), during storage.

At 4 ºC, in both conditions (with and without retarding polymer) a higher survival of *P. pentosaceus* SB83 was recorded during storage than at room temperature ($P < 0.05$).

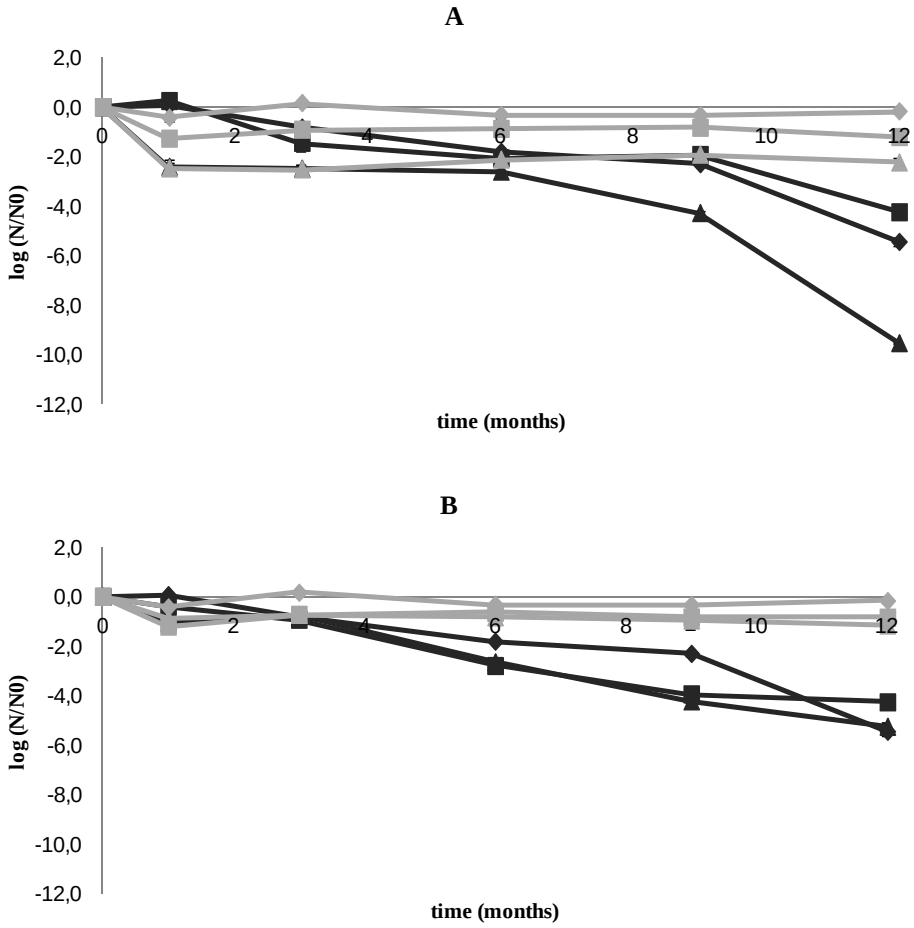

Figure 7.2. Viability of *P. pentosaceus* SB83 in preparations with (A) retarding polymer and (B) without retarding polymer during storage. The viability was examined in freeze-dried powder (♦), freeze dried powder with excipients (■), and tablets (▲) at room temperature (black lines) and at 4 ºC (grey lines). All points are means ± standard deviations.

7.4.4. Evaluation of antimicrobial activity of *Pediococcus pentosaceus* SB83 in simulated vaginal fluid

Figure 7.3. shows the antimicrobial activity *of P. pentosaceus* SB83 (in bacterial suspension, lyophilized, tablets with and without retarding polymer) against *L. monocytogenes* 2092 4b, in SVF at pH = 6.5.

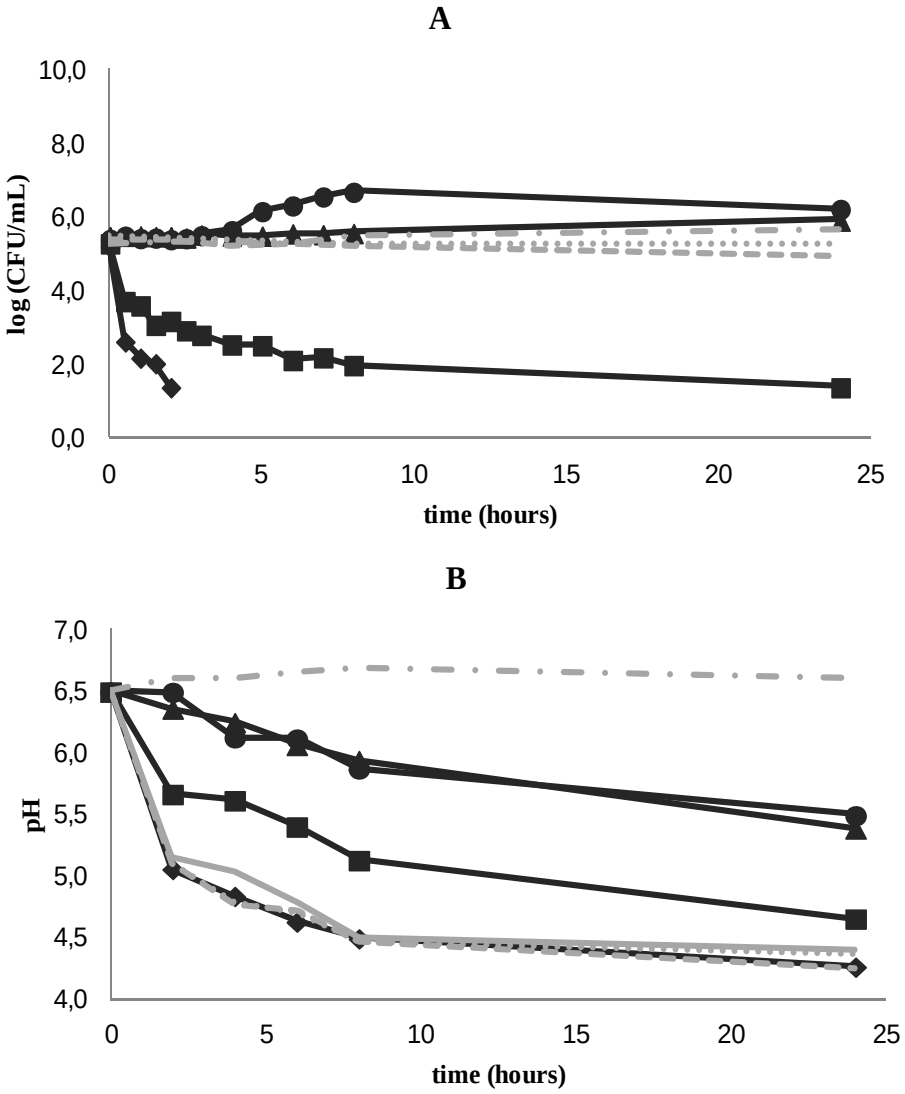

Figure 7.3. Antimicrobial activity of *P. pentosaceus* SB83 against *L. monocytogenes* 2092 4b in SVF at pH = 6.5. (A) Counts of viable cells of *L. monocytogenes* 2092 4b and (B) pH of SVF during 24 h of contact, are represented. The tested conditions were SVF inoculated with: bacterial suspension of *P. pentosaceus* SB83 and *L. monocytogenes* 2092 4b (♦); lyophilized of *P. pentosaceus* SB83 and *L. monocytogenes* 2092 4b (■); tablet of *P. pentosaceus* SB83 with retarding polymer and *L. monocytogenes* 2092 4b (▲) and tablet of *P. pentosaceus* SB83 without retarding polymer and *L. monocytogenes* 2092 4b (●). Controls were: SVF with 1 mg/mL of trypsin and inoculated with *P. pentosaceus* SB83 and *L. monocytogenes* 2092 4b (····),

SVF inoculated with a non- bacteriocinogenic LAB strain ESB67 and *L. monocytogenes* 2092 4b (– –), SVF inoculated with *P. pentosaceus* SB83 (—), SVF inoculated with *L. monocytogenes* 2092 4b (— ·). All points are means ± standard deviations.

The bacterial suspension of *P. pentosaceus* SB83 reduced *L. monocytogenes* 2092 4b below the detection limit after only 2 h. The lyophilized bacteria inhibited *Listeria* population mainly over 8 h of contact, however total inhibition (up to the limit of detection) was recorded after 24 h. The tablets did not reduce *L. monocytogenes* 2092 4b, during 24 h of experiment (P = 0.11) (Figure 7.3., graph A).

The viable counts of *P. pentosaceus* SB83 were maintained throughout the 24 h in all conditions tested (data not shown).

All conditions tested, demonstrated the ability to decrease the pH of SVF (Figure 7.3., graph B). However, the highest reduction of pH was observed for bacterial suspension, followed by the lyophilized cells. The lowest reduction of pH was observed when tablets formulations were used. No significant difference was achieved for tablets with and without retarding polymer (P = 0.94).

After obtaining these results, lyophilized bacteria were selected to evaluate the anti-listerial activity, during storage at room temperature and at 4 ºC for 12 months (Figure 7.4.).

The lyophilized bacteria when stored at room temperature, reduced totally *L. monocytogenes* 2092 4b (up to the limit of detection) until one month of storage. After 3 months, a decrease in antimicrobial capability was observed. From 3 to 12 months of storage, there was a similar behavior (P > 0.05), the pathogen was reduced in the early hours of contact with the LAB isolate, though there was an increase of *L. monocytogenes* 2092 4b after 24 h (Figure 7.4., graph A).

When storage was performed at 4 ºC, the antimicrobial activity of *P. pentosaceus* SB83 was maintained during storage ($P = 0.66$), showing only a slight decrease in antimicrobial potential after 12 months (Figure 7.4., graph D).

As previously observed, lyophilized *P. pentosaceus* SB83 was more stable at 4 ºC than room temperature (Figure 7.4., graphs B and E).

During storage of the lyophilized bacteria at room temperature, the ability of *P. pentosaceus* SB83 to reduce the pH of SVF decreased over time ($P < 0.001$) (Figure 7.4., graph C). In contrast, during storage at 4 ºC the ability to reduce the pH of SVF throughout the time of storage was observed. This capacity only decreased slightly with time ($P = 0.66$; Figure 7.4., graph F).

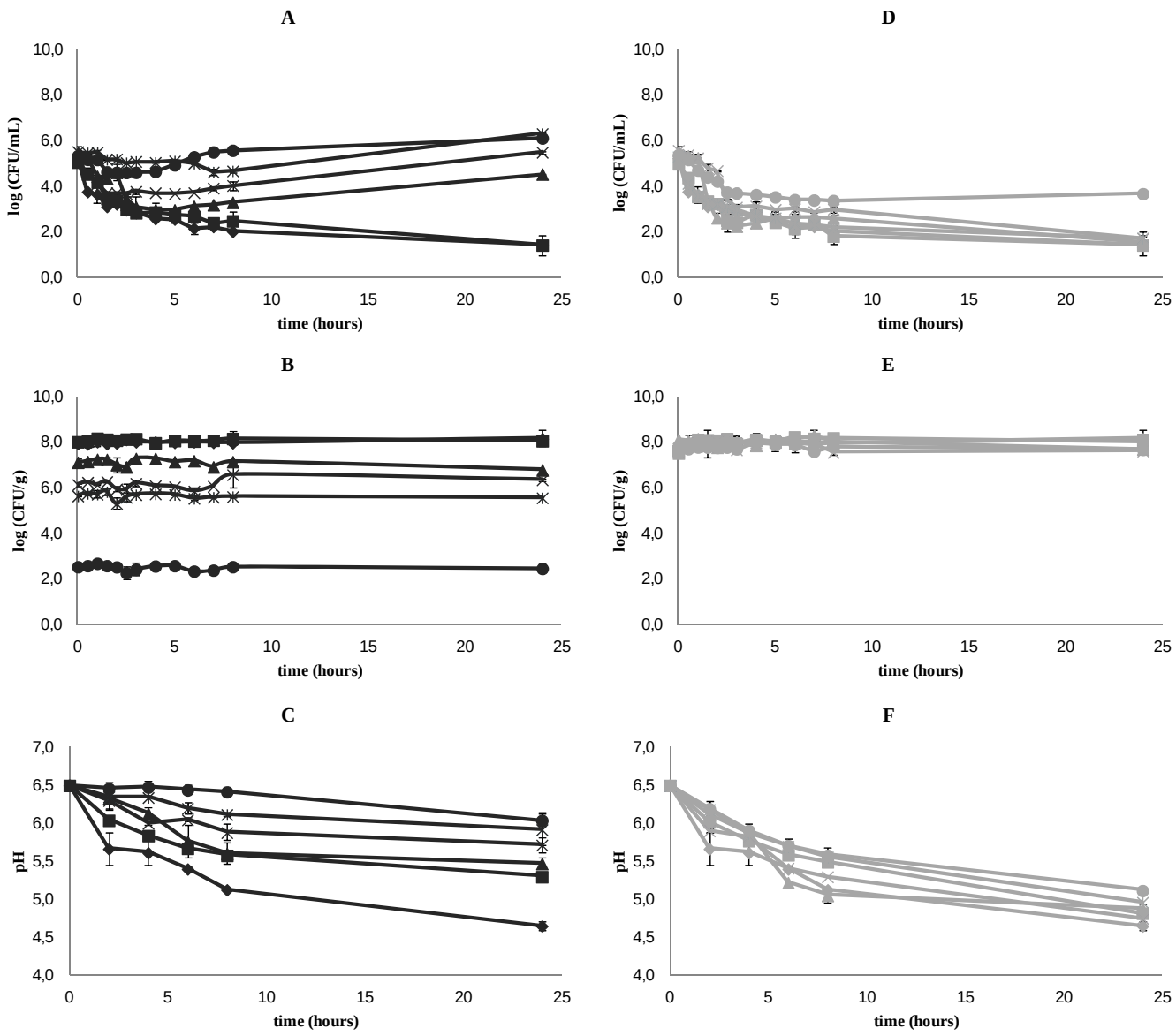

Figure 7.4. Antimicrobial activity of lyophilized of *P. pentosaceus* SB83 against *L. monocytogenes* 2092 4b in SVF at pH = 6.5, during storage at room temperature (black lines) and at 4 ºC (grey lines). (A and D) Counts of viable cells of *L. monocytogenes* 2092 4b; (B and E) counts of viable cells of *P. pentosaceus* SB83; (C and F) pH of SVF overtime are represented. These parameters were evaluated at storage time of 0 (♦), 1 (■), 3 (▲), 6 (×), 9 (ж) and 12 (●) months. All points are means ± standard deviations.

7.5. Discussion

The intra-vaginal route is normally used for the administration of drugs such as antimicrobials, labour-inducing agents, spermicidal agents, prostaglandins and steroids (Baloglu *et al.*, 2009). Accordingly, the vaginal route can be used to prevent vaginal colonization by pathogenic microorganisms in pregnant women and thereby is a method of protection of the fetus/neonate.

Based on this principle, different pharmaceutical formulations *with P. pentosaceus* SB83, a bacteriocin-producing LAB (Borges *et al.*, 2013a), were performed for subsequent vaginal administration.

In this study freeze-dried cells of *P. pentosaceus* SB83 were used, containing 10^{10} CFU/g, which remained after the production of tablets, so tabletting had no adverse effect on the viability of bacteria. Generally, the quantity used for vaginal probiotics is between 10^{8} and 10^{10} CFU in the product (Ehrström *et al.*, 2010; Larsson *et al.*, 2008; Maggi *et al.*, 2000; Mastromarino *et al.*, 2002).

The normal vaginal pH in premenopausal women is acidic, however vaginal pH can be increased due a depletion of vaginal lactobacilli (Donders *et al.*, 2007), menstruation, unprotected sexual intercourse with the deposition of semen (Tevi-Bénissan *et al.*, 1997) and vaginal medications. Therefore, we tested the release of *P. pentosaceus* SB83 in SVF at different values of pH (4.2, 5.5 and 6.5). The pH influenced the release of bacteria from tablets. Tablets with retarding polymer as well as tablets without retarding polymer, released fewer cells of *P. pentosaceus* SB83 at pH 4.2.

The presence of retarding polymer affected the total numbers of bacteria released. The polymer used (HPMC) retained *P. pentosaceus* SB83 inside the tablets, releasing a maximum of 10^{8} CFU/g. However, this release occurred in the early hours of contact with SVF, such as in tablets without the retarding polymer. Fazeli *et al*. (2006)

performed an assay with tablets with HPMC and concluded that a high initial bacterial load release occur during the first 15 min in deionized sterile water, however, the tablets showed a continuous bacterial release during 5.75 h.

The tablets and lyophilized powder were stored during 12 months, in order to determine their stability. There was an effect of temperature on viability of cells during storage; *P. pentosaceus* SB83 showed a higher survival rate at 4 ºC than at room temperature. According to other studies, freeze-dried bacteria are more stable at low temperatures than at room temperature (Kaewnopparat and Kaewnopparat, 2009; Klayraung *et al.*, 2009). It was reported that milk promotes survival at low temperature by stabilizing the cell membrane constituents and forming a protective coating on the cell wall proteins (see review Carvalho *et al.*, 2004).

At 4 ºC the viability of *P. pentosaceus* SB83 was maintained at a high level in all formulations tested, for at least 12 months. Fazeli *et al*. (2006) also demonstrated that slow-release tablets were stable at 4 ºC during 6 months of storage. Maggi *et al.* (2000) tested the viability of 10 lactobacilli strains in freeze-dried powder and tablets and have shown that stability depends on the bacterial strain and also on the polymer used as a retarding compound. In their study, four strains of lactobacilli maintained a high viability for 18 months at 4 ºC.

While observing an elevated survival of bacteria during storage, it is important to confirm that the antimicrobial characteristics are also maintained. In previous studies, our group characterized the bacteriocin produced by *P. pentosaceus* SB83 in MRS medium (Borges *et al.*, 2013a) and tested the antimicrobial activity of *P. pentosaceus* SB83 after exposure to SVF at pH 4.2 after 24h (Borges *et al.*, 2013b). However, it is relevant to evaluate the production and effectiveness of bacteriocin production by the LAB after storage, in SVF, in case of vaginal colonization by pathogens (elevated pH).

The pathogen selected to assess these parameters was *L. monocytogenes*. This bacterium can be acquired via vertical transmission from mother to fetus/neonate through the placenta or birth canal (Delgado, 2008, Posfay-Barbe and Wald, 2009) and can lead to pre-term labour, chorioamnionitis, spontaneous abortion, stillbirth and neonatal infection (DiMaio, 2000).

In experiments to determine inhibitory activity of stored cells of *P. pentosaceus* SB83 SVF at pH of 6.5 was used, because it was demonstrated previously that clinical strains of *L. monocytogenes* are inhibited by the normal vaginal pH for 48 h, but may proliferate when the pH increases (Borges *et al.*, 2011).

The bacterial suspension of *P. pentosaceus* SB83 reduced *L. monocytogenes* 2092 4b below the detection limit after only 2 h (reduction of approximately 4.0 log). The pH of SVF was decreased to normal vaginal pH (4.2) in 24 h.

The lyophilized bacteria showed a total inhibition of the pathogen after 24 h of contact. The pH of SVF decreased to values of 4.5 in 24 h.

The tablets did not reduce counts of *L. monocytogenes* 2092 4b, although the bacteria in the tablets were able to decrease the pH to values of 5.5 in 24 h. These results showed that the activity of the bacteriocin SB83 was affected by the excipients used in production of tablets, probably by its adsorption to the excipients. It has been reported that some compounds commonly used as pharmaceutical excipients and/or starter culture cryoprotectants can alter or completely inhibit the activity of bacteriocins (Zárate and Nader-Macias, 2006). The reduction in pH was rather less by cells in tablets than by lyophilized cells, this also suggests that the tabletted cells are rather less metabolically active than lyophilized cells.

In the controls used (SVF with 1 mg/mL of trypsin and inoculated with *P. pentosaceus* SB83 and *L. monocytogenes* 2092 4b; SVF inoculated with a non-bacteriocinogenic

LAB strain ESB67 and *L. monocytogenes* 2092 4b), there was no inhibition of pathogen, while there was a decrease of pH. This suggests that the antimicrobial activity observed in bacterial suspension and lyophilized in SVF was due to the production of bacteriocin SB83.

The studies performed on the production of bacteriocins in the vaginal environment are very few. According to the study by Tomás and Nader-Macías (2007), *Lact. salivarius* CRL 1328 was able to grow in SVF at 4.25, but that bacteriocin production was minimal, only 40 AU/mL.

The lyophilized powder demonstrated antimicrobial activity, therefore this formulation was selected to evaluate the anti-listerial activity during the 12 months of storage. In pharmaceutical products it is important verify the stability of the bioactive compounds and the maintenance of the biological activity during long periods of storage.

When stored at room temperature, lyophilized cells inhibited totally the pathogen (up to the limit of detection) up to one month of storage. After this time, there was a decrease in antimicrobial potential that may be caused by diminishing numbers of viable cells of *P. pentosaseus* SB83 during storage at room temperature. This decrease in the number of cells could also have influenced the reduction of pH of SVF, the production of acid is lower with storage time of *P. pentosaceus* SB83 cells.

When lyophilized cells were stored at 4 ºC, *P. pentosaceus* SB83 maintained their viability, consequently the anti-listerial activity and the capacity to produce acid was observed throughout the time of storage. The bacteriocin SB83 remained active during at least 12 months, in spite of a decrease that occurred in antimicrobial activity at the twelfth month of storage.

Zárate and Nader-Macias (2006) evaluated the bacteriocinogenic activity of *L. salivarius* CRL 1328 after lyophilization (with different protective substances, such as

lactose, skim milk, ascorbic acid and combinations of them) during storage at 5 ºC. It was demonstrated that bacteriocin synthesis was not affected by the lyophilization, but a slight decrease of activity was observed during storage in cells lyophilized with lactose and completely abolished in cells lyophilized with ascorbic acid.

Vera Pingitore *et al*. (2012) tested the activity of a lyophilized bacteriocin (salivaricin) during storage at -25 ºC, 4 ºC and 25 ºC for 18 months. The antimicrobial activity of salivaricin was dependent mainly on temperature, and also on the time of storage and protectant used to lyophilize. The stability of salivaricin was higher at low temperatures than 25 ºC and decreased during the time of storage.

To our knowledge, this is the first study where the bacteriocin production of lyophilized bacteria was tested against *L. monocytogenes*, under the conditions of vaginal fluid and throughout 12 months of storage.

The assays for antimicrobial activity were performed against *L. monocytogenes*; nevertheless bacteriocin SB83 also inhibits the growth of *Enterococcus* spp. (Borges *et al.*, 2013a); thus the lyophilized cells may also be used to prevent UTI caused by this pathogen.

Pediococcus pentosaceus SB83 showed the ability to reduce the pH of SVF, which is relevant because in addition to inhibiting those pathogens previously mentioned, this LAB has the capability of preventing colonization by other vaginal pathogens by reduction of vaginal pH acid; one study reported the inhibition of GBS in SVF at pH 4.2 (Borges *et al.*, 2012). Moreover, the maintenance of the acidic vaginal pH will allow the proliferation of the vaginal *Lactobacillus* spp. belonging to the normal microbiota of the vaginal tract. Hemalatha *et al.* (2012) demonstrated that a probiotic lactobacilli tablet was found to be better than a pH lowering vaginal tablet, in preventing BV in healthy subjects.

7.6. Conclusions

The better formulation of *P. pentosaceus* SB83 is the lyophilized powder which may be administered vaginally as a washing solution. Based on the tests carried out, the product must be stored at 4 ºC and can be used for a period of time of 9 months. If storage is performed until this time, the characteristics of cells are maintained, i.e., the number of viable cells, the production of an active bacteriocin and the ability to reduce vaginal pH. Further assays need to be performed, including adhesion to vaginal epithelial cells and clinical trials, to determine the frequency of administration and the effectiveness *in vivo* of the lyophilized powder of *P. pentosaceus* SB83. Other formulations can be tested using lyophilized bacteria, including tablets with suitable excipients, ovules and gels.

8. General discussion

Infections in the genital tract can affect pregnant women and cause premature birth, neonatal infection, congenital abnormalities, among other serious consequences. Therefore, it is important to understand the behavior of the pathogens, *L. monocytogenes* and GBS, in vaginal conditions in order to evaluate their pathogenesis and ascending infection.

The pH of vaginal secretions influences the growth of *L. monocytogenes* and GBS. At normal vaginal pH (pH 4.2) there was a greater reduction in viable cells than at higher pH values (pH 5.5 and 6.5). Therefore, the vaginal acidity inhibits these pathogens, but when vaginal pH increases due to various factors (a depletion of vaginal lactobacilli, menstruation, unprotected sexual intercourse with the deposition of semen, vaginal medications), these pathogens can survive and proliferate.

Moreover, *L. monocytogenes* and GBS have the ability to form biofilms in SVF at different pH values. Biofilm production plays an important role in the stress resistance of bacteria and diagnoses in the presence of biofilm are difficult. Prenatal culture-based screening for vaginal and rectal GBS colonization of all pregnant women at 35 to 37 weeks gestation is universally recommended, however the detection of *L. monocytogenes* is not done. Thus, vaginal colonization by *L. monocytogenes* is underestimated.

When urogenital infections are detected, treatment is with antibiotics which have the undesirable effects of decreasing the number of lactobacilli, do not restore the urinary tract natural barrier to infections and increase the risk of developing drug resistance. The use of vaginal probiotics could be a very attractive alternative as a preventive or therapeutic.

Pediococcus spp. showed antimicrobial activity against *L. monocytogenes*, and their antimicrobial activity was shown to be mainly caused by production of a bacteriocin. These isolates of *Pediococcus* spp. have two fields of action, (a) inhibit directly *L. monocytogenes* by action of a bacteriocin and (b) maintain the acidic vaginal pH, which will prevent colonization and multiplication by other pathogenic microrganisms, including GBS. *Pediococcus* spp. can survive in SVF and all isolates demonstrated anti-listerial activity after exposure to SVF.

Pediococcus pentosaceus SB83 showed a greater bacteriocinogenic activity compared with other isolates of *Pediococcus* spp. and this strain maintained viability in SVF, showing only a slight decrease after 48 h exposure (0.3 cycle log). *Pediococcus pentosaceus* SB83 did not produce virulence factors such as gelatinase, lipase and DNase, hemolytic activity, nor show the presence of virulence genes (genes of surface adhesion, aggregation protein, cytolysin and extracellular metallo-endopeptidase). This strain was considered intrinsically resistant to tetracycline and vancomycin. Intrinsic resistance is not horizontally transferable, and poses no risk in non-pathogenic bacteria. *Pediococcus pentosaceus* SB83 produced a biofilm in SVF at normal vaginal pH and higher pH values. *Pediococcus pentosaceus* SB83 biofilms also serve as a protective layer against colonization by other pathogenic bacteria.

Bacteriocins have been considered promising natural entities for application in food preservation or medical treatment due to their effective antimicrobial activity against several pathogens, and their metabolic properties. Bacteriocin SB83 inhibits *L. monocytogenes* and also inhibited the growth of *Ent. faecalis*, *Ent. faecium* and *B. subtilis*. The inhibition of *Enterococcus* spp. would be interesting in the prevention of urinary tract infections. Bacteriocin SB83 is stable in conditions of vaginal fluid (components and pH), is resistant to different detergents and does not inhibit the vaginal

lactic acid bacteria. These characteristics are important for application of this bacteriocin in the vaginal tract.

The better formulation of *P. pentosaceus* SB83 is the lyophilized powder which may be administered vaginally for example, as a washing solution.

The lyophilized bacteria showed a total inhibition of the *L. monocytogenes* after 24 h of contact. The pH of SVF decreased from 6.5 to 4.5, in 24 h.

9. Main conclusion

Pediococcus pentosaceus SB83 has the potential to be used as a vaginal probiotic, to prevent colonization of *L. monocytogenes* in pregnant women and consequently to reduce neonatal infections. Natural antimicrobials, while inhibiting growth of pathogens, will help the survival and promote the growth of the native *Lactobacillus* spp., thus supporting the natural defenses against pathogenic organisms.

The lyophilized powder was shown to be the best formulation to guarantee the antimicrobial activity of the cells during storage. The lyophilized must be stored at 4 ºC and can be used for a period up to 9 months. If storage is performed until this time, the characteristics of cells are maintained, i.e. the number of viable cells, the production of an active bacteriocin and the ability to reduce vaginal pH.

10. Proposals for Future Work

The present study demonstrated the possibility of using *P. pentosaceus* SB83 for prevention of neonatal listeriosis. The following suggestions for future wok can be proposed:

- Adhesion assays to vaginal epithelial cells are required for the use of the potential probiotic.

- Determine the presence of potentially transferable antibiotic resistances in the potential probiotic.

- Purify the bacteriocin and determine the aminoacid sequence in order to make a characterization more complete.

- Test the potential use of purified bacteriocin.

- Assess synergistic combinations, for instance, mixed-culture bacteriocinogenic LAB.

- To obtain evidence of the preventive/curative role of the probiotic, studies *in vivo* will be necessary to develop correct formulation relative to the numbers of bacteria, viability, administration frequency and efficacy.

- Test other formulations, for example ovules and gels.

11. References

Abrams, D., Barbosa, J., Albano, H., Silva, J., Gibbs, P.A., Teixeira, P. 2011. Characterization of bacPPK34 a bacteriocin produced by *Pediococcus pentosaceus* strain K34 isolated from "Alheira". Food Control 22, 940-946.

Ahmed, Z., Wang, Y., Cheng, Q., Imran, M. 2010. *Lactobacillus acidophilus* bacteriocin, from production to their application: An overview. African Journal of Biotechnology 9, 2843-2850.

Albano, H., Todorov, S.D., van Reenen, C.A., Hogg, T., Dicks, L.M.T., Teixeira, P. 2007. Characterization of two bacteriocins produced by *Pediococcus acidilactici* isolated from "Alheira", a fermented sausage traditionally produced in Portugal. International Journal of Food Microbiology 116, 239-247.

Almeida, G.N., Gibbs, P.A., Hogg, T.A., Teixeira, P.C. 2006. Listeriosis in Portugal: an existing but under reported infection. BMC Infectious Diseases 6, 153-157.

Ammor, M.S., Floréz, A.B., Mayo, B. 2007. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiology 24, 559-570.

Anastasiadou, S., Papagianni, M., Filiousis, G., Ambrosiadis, I., Koidis, P. 2008. Pediocin SA-1, an antimicrobial peptide from *Pediococcus acidilactici* NRRL B5627: Production conditions, purification and characterization. Bioresource Technology 99, 5384-5390.

Andrighetto, C., Zampese, L., Lombardi, A. 2001. RAPD-PCR characterization of lactobacilli isolated from artisanal meat plants and traditional fermented sausages of Veneto region (Italy). Letters in Applied Microbiology 33, 26-30.

Antonio, M.A.D., Hawes, S.E., Hillier, S.L. 1999. The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. Journal of Infectious Diseases, 1950-1956.

Anukam, K.C. 2007. The potential role of probiotics in reducing poverty-associated infections in developing countries. Journal of Infection in Developing Countries 1, 81-83.

Anukam, K.C., Osazuwa, E.O., Ahonkhai, I., Reid, G. 2006. *Lactobacillus* vaginal microbiota of women attending a reproductive health care service in Benin City, Nigeria. Sexually Transmitted Diseases 33, 59-62.

Aroutcheva, A., Gariti, D., Simon, M., Shott, S., Faro, J., Simoes, J.A., Gurguis, A., Faro, S. 2001. Defense factors of vaginal lactobacilli. American Journal of Obstetrics and Gynecology 185, 375-379.

Aslim, B., Kilic, E. 2006. Some probiotic properties of vaginal lactobacilli isolated from healthy women. Japanese Journal of Infectious Diseases 59, 249-253.

Baloglu, E., Senyigit, Z.A., Karavana, S.Y., Bernkop-Schnürch, A. 2009. Strategies to prolong the intravaginal residence time of drug delivery systems. Journal of Pharmacy and Pharmaceutical Sciences 12, 312-336.

Barbosa, J., Gibbs, P.A., Teixeira, P. 2010. Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal. Food Control 21, 651-656.

Barefoot, S.F., Klaenhammer, T.R. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Applied and Environmental Microbiology and Molecular Biology Reviews 45, 1808-1815.

Barrons, R., Tassone, D. 2008. Use of *Lactobacillus* probiotics for bacterial genitourinary infections in women: A review. Clinical Therapeutics 30, 453-468.

Ben Omar, N., Castro, A., Lucas, R., Abriouel, H., Yousif, N.M., Franz, C.M., Holzapfel, W.H., Pérez-Pulido, R., Martínez-Cañamero, M., Gálvez, A. 2004

Functional and safety aspects of enterococci isolated from different Spanish foods. Systematic and Applied Microbiology 27, 118-130.

Bolton, M., Van Der Straten, A., Cohen, C. 2008. Probiotics: potential to prevent HIV and sexually transmitted infections in women. Sexually Transmitted Diseases 35, 214-225.

Bonaventura, G.D., Piccolomini, R., Paludi, D., D'Orio, V., Vergara, A., Conter, M., Ianieri, A. 2008. Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. Journal of Applied Microbiolology 104, 1552-1561.

Borges, S., Silva, J., Teixeira, P. 2012. Survival and biofilm formation by Group B streptococci in simulated vaginal fluid at different pHs. Antonie van Leeuwenhoek 101, 677-682.

Borges, S., Barbosa, J., Silva, J., Teixeira, P. 2013a. Characterization of a bacteriocin of *Pediococcus pentosaceus* SB83 and its potential for vaginal application. Anti-Infective Agents 11(2) (in press).

Borges, S., Barbosa, J., Silva, J., Teixeira, P. 2013b. Evaluation of characteristics of *Pediococcus* spp. to be used as a vaginal probiotic. Journal of Applied Microbiology , doi: 10.1111/jam.12232.

Borges, S.F., Silva, J.G.L., Teixeira, P.C.M. 2011. Survival and biofilm formation of *Listeria monocytogenes* in simulated vaginal fluid: infuence of pH and strain origin. FEMS Immunology and Medical Microbiology 62, 315-320.

Boris, S., Barbés, C. 2000. Role played by lactobacilli in controlling the population of vaginal pathogens. Microbes and Infection 2, 543-546.

Boris, S., Suárez, J.E., Vázquez, F., Barbés, C. 1998. Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infection and Immunity 66, 1985-1989.

Boskey, E.R., Telsch, K.M., Whaley, K.J., Moench, T.R., Cone, R.A. 1999. Acid production by vaginal flora in vitro is consistent with the rate and extent of vaginal acidification. Infection and Immunity 67, 5170-5175.

Brolazo, E.M., Simões, J.A., Nader, M.E.F., Tomás, M.S.J., Gregoracci, G.B., Marconi, C. 2009. Prevalência e caracterização de espécies de lactobacilos vaginais em mulheres em idade reprodutiva sem vulvovaginites. Revista Brasileira de Ginecologia e Obstetrícia 31, 189-195.

Burton, J.P., Reid, G. 2002. Evaluation of the bacterial vaginal flora of 20 postmenopausal women by direct (Nugent score) and molecular (polymerase chain reaction and denaturing gradient gel electrophoresis) techniques. Journal of Infectious Diseases 186, 1770-1780.

Burton, J.P., Cadieux, P.A., Reid, G. 2003. Improved understanding of the bacterial vaginal microbiota of women before and after probiotic instillation. Applied and Environmental Microbiology 69, 97-101.

Caillouette, J.C., Sharp, C.F., Zimmerman, G.J., Roy, S. 1997. Vaginal pH as a marker for bacterial pathogens and menopausal status. American Journal of Obstetrics & Gynecology 176, 1270-1277.

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X., Gibbs, P. 2004. Relevant factors for the preparation of freeze-dried lactic acid bacteria. International Dairy Journal 14, 835-847.

Chae, M.S., Schraft, H. 2000. Comparative evaluation of adhesion and biofilm formation of different *Listeria monocytogenes* strains. International Journal of Food Microbiology 62, 103-111.

Charlier, C., Cretenet, M., Even, S., Le Loir, Y. 2009. Interactions between *Staphylococcus aureus* and lactic acid bacteria: An old story with new perspectives. International Journal of Food Microbiology 131, 30-39.

Chen, S., Lu, F.L., Lee, P., Lu, C., Chen, C., Chou, H., Tsao, P., Hsieh, W. 2007. Neonatal listeriosis. Journal of the Formososan Medical Association 106, 161-164.

Conti, C., Malacrino, C., Mastromarino, P. 2009. Inhibition of herpes simplex virus type 2 by vaginal lactobacilli. Journal of Physiology and Pharmacology 60, 19-26.

Coudeyras, S., Jugie, G., Vermerie, M., Forestier, C. 2008. Adhesion of human probiotic *Lactobacillus rhamnosus* to cervical and vaginal cells and interaction with vaginosis-associated pathogens. Infectious Diseases in Obstetrics and Gynecology 2008, 1-5.

Courvalin, P. 2006. Antibiotic resistance: the pros and cons of probiotics. Digestive and Liver Disease 38, S261-S265.

Cribby, S., Taylor, M., Reid, G. 2008. Vaginal microbiota and the use of probiotics. Interdisciplinary Perspectives on Infectious Diseases 2008, 1-9.

Czaja, C.A., Stapleton, A.E., Yarova-Yarovaya, Y., Stamm, W.E. 2007. Phase I trial of a *Lactobacillus crispatus* vaginal suppository for prevention of recurrent urinary tract infection in women. Infectious Diseases in Obstetrics and Gynecology 2007, 1-8.

Danielsen, M., Simpson, P.J., O'Connor, E.B., Ross, R.P., Stanton, C. 2007. Susceptibility of *Pediococcus* spp. to antimicrobial agents. Journal of Applied Microbiology 102, 384-389.

Delgado, A.R. 2008. Listeriosis in Pregnancy. Journal of Midwifery & Women's Health 53, 255-259.

Dezwaan, D.C., Mequio, M.J., Littell, J.S., Allen, J.P., Rossbach, S., Pybus, V. 2007. Purification and characterization of enterocin 62-6, a two-peptide bacteriocin produced by a vaginal strain of *Enterococcus faecium*: Potential significance in bacterial vaginosis. Microbial Ecology in Health and Disease 19, 241-250.

Dicks, L.M.T., Silvester, M., Lawson, P.A., Collins, M.D. 2000. *Lactobacillus fornicalis* sp. nov., isolated from the posterior fornix of the human vagina. International Journal of Systematic and Evolutionary Microbiology 50, 1253-1258.

DiMaio, H. 2000. Listeria infection in women. Primary Care Update for OB/GYNS 7, 40-45.

Döderlein, A. 1892. Das scheidensekret und seine bedeutung fur puerperalfieber. Zentralblatt fur Bakteriology 11, 699.

Donati, L., Di Vico, A., Nucci, M., Quagliozzi, L., Spagnuolo, T., Labianca, A., Bracaglia, M., Ianniello, F., Caruso, A., Paradisi, G. 2010. Vaginal microbial flora and outcome of pregnancy. Archives of Gynecology and Obstetrics 281, 589-600.

Donders, G.G., Vereecken, A., Bosmans, E., Dekeersmaecker, A., Salembier, G., Spitz, B. 2002. Definition of a type of abnormal vaginal flora that is distinct from bacterial vaginosis: aerobic vaginitis. BJOG 109, 34-43.

Donders, G.G.G., Caeyers, T., Tydhof, P., Riphagen, I., van den Bosch, T., Bellen, G. 2007. Comparison of two types of dipsticks to measure vaginal pH in clinical

practice. European Journal of Obstetrics & Gynecology and Reproductive Biology 134, 220-224.

Dover, S.E., Aroutcheva, A.A., Faro, S., Chikindas, M.L. 2007. Safety study of an antimicrobial peptide lactocin 160, produced by the vaginal *Lactobacillus rhamnosus*. Infectious Diseases in Obstetrics and Gynecology 2007, 1-6.

Dover, S.E., Aroutcheva, A.A., Faro, S., Chikindas, M.L. 2008. Natural antimicrobials and their role in vaginal health: a short review. International Journal of Probiotics and Prebiotics. 3, 219-230.

Drago, L., de Vecchi, E., Nicola, L., Zucchetti, E., Gismondo, M.R., Vicariotto, F. 2007. Activity of a *Lactobacillus acidophilus*–Based Douche for the Treatment of Bacterial Vaginosis. The Journal of Alternative and Complementary Medicine 13, 435-438.

Drider, D., Fimland, G., Héchard, Y., McMullen, L., Prévost, H. 2006. The continuing story of class IIa bacteriocins. Microbiology and Molecular Biology Reviews 70, 564-582.

Eaton, T.J., Gasson, M.J. 2001. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Applied and Environmental Microbiology 67, 1628-1635.

Ehrström, S., Daroczy, K., Rylander, E., Samuelsson, C., Johannesson, U., Anzén, B., Påhlson, C. 2010. Lactic acid bacteria colonization and clinical outcome after probiotic supplementation in conventionally treated bacterial vaginosis and vulvovaginal candidiasis. Microbes and Infection 12, 691-699.

Eriksson, K., Carlsson, B., Forsum, U., Larsson, P.-G. 2005. A double-blind treatment study of bacterial vaginosis with normal vaginal lactobacilli after an open

treatment with vaginal clindamycin ovules. Acta Dermato Venereologica 85, 42-46.

European Food Safety Authority (EFSA). Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance (question no. EFSA-Q-2008-004), adopted on 18 June 2008. EFSA Journal 2008; 732,1-15.

Falagas, M.E., Betsi, G.I., Athanasiou, S. 2007. Probiotics for the treatment of women with bacterial vaginosis. Clinical Microbiology and Infection 13, 657-664.

Farage, M.A., Miller, K.W., Sobel, J.D. 2010. Dynamics of the vaginal ecosystem-hormonal influences. Infectious Diseases: Research and Treatment 3, 1-15.

Fazeli, M.R., Toliyat, T., Samadi, N., Hajjaran, S., Jamalifar, H. 2006. Viability of lactobacillus acidophilus in various vaginal tablet formulations. DARU 14, 172-177.

Fernandez, M., Carol, J.B. 1999. Group B streptococcal infections. Seminars in Pediatric Infections Diseases 10, 104-110.

Fraga, M., Perelmuter, K., Delucchi, L., Cidade, E., Zunino, P. 2008. Vaginal lactic acid bacteria in the mare: evaluation of the probiotic potential of native *Lactobacillus* spp. and *Enterococcus* spp. strains. Antonie van Leeuwenhoek 93, 71-78.

Ganatra, H.A., Zaidi, A.K.M. 2010. Neonatal Infections in the Developing World. Seminars in Perinatology 34, 416-425.

Garcia-Closas, M., Herrero, R., Bratti, C., Hildesheim, A., Sherman, M.E., Morera, L.A., Schiffman, M. 1999. Epidemiologic determinants of vaginal pH. American Journal of Obstetrics & Gynecology 180, 1060-1066.

Garg, S., Goldman, D., Krumme, M., Rohan, L.C., Smoot, S., Friend, D.R. 2010. Advances in development, scale-up and manufacturing of microbicide gels, films, and tablets. Antiviral Research 88S, S19-S29.

Geshnizgani, A.M., Onderdonk, A.B. 1992. Defined medium simulating genital tract secretions for growth of vaginal microflora. Journal of Clinical Microbiology 30, 1323-1326.

Gibbs, R.S., Schrag, S., Schuchat, A. 2004. Perinatal infections due to Group B streptococci. Obstetrics & Gynecology 104, 1062-1076.

Gillor, O., Nigro, L., Riley, M. 2005. Genetically engineered bacteriocins and their potential as the next generation of antimicrobials. Current Pharmaceutical Design 11, 1067-1075.

Guerrieri, E., de Niederhäusern, S., Messi, P., Sabia, C., Iseppi, R., Anacarso, I., Bondi, M. 2009. Use of lactic acid bacteria (LAB) biofilms for the control of *Listeria monocytogene*s in a small-scale model. Food Control 20, 861–865.

Gurira, O.Z., Buys, E.M. 2005. Characterization and antimicrobial activity of *Pediococcus* species isolated from South African farm-style cheese. Food Microbiology 22, 159-168.

Harvey, J., Keenan, K.P., Gilmour, A. 2007. Assessing biofilm formation by *Listeria monocytogenes* strains. Food Microbiology 24, 380-392.

Hay, P. 2005. Genito-urinary infections in pregnancy. Women's Health Med 2, 47-50.

Hemalatha, R., Mastromarino, P., Ramalaxmi, B.A., Balakrishna, N.V., Sesikeran, B. 2012. Effectiveness of vaginal tablets containing lactobacilli versus pH tablets on vaginal health and inflammatory cytokines: a randomized, double-blind study. European Journal of Clinical Microbiology & Infectious Diseases 31, 3097-3105.

Hickey, R.J., Zhou, X., Pierson, J.D., Ravel, J., Forney, L.J. 2012. Understanding vaginal microbiome complexity from an ecological perspective. Translational Research 160, 267-282.

Hood, S.K., Zottola, E.A. 1997. Adherence to stainless steel by foodborne microorganisms during growth in model food systems. International Journal of Food Microbiology 37, 145-153.

Huang, Y., Luo, Y., Zhai, Z., Zhang, H., Yang, C., Tian, H., Li, Z., Feng, J., Liu, H., Hao, Y. 2009. Characterization and application of an anti-*Listeria* bacteriocin produced by *Pediococcus pentosaceus* 05-10 isolated from Sichuan Pickle, a traditionally fermented vegetable product from China. Food Control 20, 1030-1035.

Hussain, A., Ahsan, F. 2005. The vagina as a route for systemic drug delivery. Journal of Controlled Release 103, 301-313.

Iannitti, T., Palmieri, B. 2010. Therapeutical use of probiotic formulations in clinical practice. Clinical Nutrition 29, 701-725.

Janakiraman, V. 2008. Listeriosis in pregnancy: diagnosis, treatment and prevention. Reviews in Obstetrics and Gynecology 1, 179-185.

Jeffery, H.E. 1996. Perinatal group B streptococcal infection: a significant public health problem. Seminars in Neonatology 1, 77-89.

Jin, L., Tao, L., Pavlova, S.I., So, J.-S., Kiwanuka, N., Namukwaya, Z., Saberbein, B.A., Wawer, M. 2007. Species diversity and relative abundance of vaginal lactic acid bacteria from women in Uganda and Korea. Journal of Applied Microbiology 102, 1107-1115.

Jonganurakkun, B., Wang, Q., Xu, S.H., Tada, Y., Minamida, K., Yasokawa, D., Sugi, M., Hara, H., Asano, K. 2008. *Pediococcus pentosaceus* NB-17 for probiotic use. Journal of Bioscience and Bioengineering 106, 69-73.

Juarez Tomás, M.S., Bru, E., Wiese, B., de Ruiz Holgado, A.A.P., Nader-Macías, M.E. 2002. Influence of pH, temperature and culture media on the growth and

bacteriocin production by vaginal *Lactobacillus salivarius* CRL 1328. Journal of Applied Microbiology 93, 714-724.

Kaewnopparat, S., Kaewnopparat, N. 2009. Formulation and evaluation of vaginal suppositories containing *Lactobacillus*. World Academy of Science, Engineering and Technology 55, 25-28.

Kale, V., Trivedi, R., Muley, P. 2008. Proposed design of a dissolution apparatus for vaginal formulations containing probiotics. Dissolution Technologies 15, 27-29.

Karaoğlu, Ş.A., Aydin, F., Kiliç, S.S., Kiliç, A.O. 2002. Antimicrobial activity and characteristics of bacteriocins produced by vaginal lactobacilli. Turkish Journal of Medical Sciences 33, 7-13.

Kaur, H., Kumar, P., Ray, P., Kaur, J., Chakraborti, A. 2009. Biofilm formation in clinical isolates of group B streptococci from north India. Microbial Pathogenesis 46, 321-327.

Kelly, M.C., Mequio, M.J., Pybus, V. 2003. Inhibition of vaginal lactobacilli by a bacteriocin-like inhibitor produced by *Enterococcus faecium* 62-6: potential significance for bacterial vaginosis. Infectious Diseases in Obstetrics and Gynecology 11, 147-156.

Kim, Y.H., Kim, C.H., Cho, M.K., Na, J.H., Song, T.B., Oh, J.S. 2006. Hydrogen peroxide-producing lactobacilli in the vaginal flora of pregnant women with preterm labor with intact membranes. International Journal of Gynecology and Obstetrics 93, 22-27.

Kingcha, Y., Tosukhowong, A., Zendo, T., Roytrakul, S., Luxananil, P., Chareonpornsook, K., Valyasevi, R., Sonomoto, K., Visessanguan, W. 2012. Anti-listeria activity of *Pediococcus pentosaceus* BCC 3772 and application as

starter culture for Nham, a traditional fermented pork sausage. Food Control 25, 190-196.

Klare, I., Konstabel, C., Werner, G., Huys, G., Vankerckhoven, V., Kahlmeter, G., Hildebrandt, B., Müller-Bertling, S., Witte, W., Goossens, H. 2007. Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. Journal of Antimicrobial Chemotherapy 59, 900-912.

Klayraung, S., Viernstein, H., Okonogi, S. 2009. Development of tablets containing probiotics: Effects of formulation and processing parameters on bacterial viability. International Journal of Pharmaceutics 370, 54-60.

Klebanoff, S.J., Watts, D.H., Mehlin, C., Headley, C.M. 1999. Lactobacilli and vaginal host defense: activation of the human immunodeficiency virus type 1 long terminal repeat, cytokine production, and NF-kB. Journal of Infectious Diseases 179, 653-660.

Konto-Ghiorghi, Y., Mairey, E., Mallet, A., Duménil, G., Caliot, E., Trieu-Cuot, P., Dramsi, S. 2009. Dual role for pilus in adherence to epithelial cells and biofilm formation in *Streptococcus agalactiae*. PLoS Pathogens 5, e1000422.

Kubota, H., Senda, S., Nomura, N., Tokuda, H., Uchiyama, H. 2008. Biofilm formation by Lactic Acid Bacteria and resistance to environmental stress. Journal of Bioscience and Bioengineering 106, 381-386.

Larsson, P.-G., Stray-Pedersen, B., Ryttig, K.R., Larsen, S. 2008. Human lactobacilli as supplementation of clindamycin to patients with bacterial vaginosis reduce the recurrence rate; a 6-month, double-blind, randomized, placebo-controlled study. BMC Women's Health 8:3.

Lecuit, M. 2007. Human listeriosis and animal models. Microbes and Infection 9, 1216-1225.

Lindsay, D., von Holy, A. 2006. Bacterial biofilms within the clinical setting: what healthcare professionals should know. Journal of Hospital Infection 64, 313-325.

Locksmith, G., Duff, P. 2001. Infection, antibiotics, and preterm delivery. Seminars in Perinatology 25, 295-309.

Ma, B., Forney, L.J., Ravel, J. 2012. Vaginal microbiome: rethinking health and disease. Annual Review of Microbiology 66, 371-389.

Maggi, L., Mastromarino, P., Macchia, S., Brigidi, P., Pirovano, F., Matteuzzi, D., Conte, U. 2000. Technological and biological evaluation of tablets containing different strains of lactobacilli for vaginal administration. European Journal of Pharmaceutics and Biopharmaceutics 50, 389-395.

Mahmoud, E.A., Svensson, L.O., Olsson, S.E., Mardh, P.A. 1995. Antichlamydial activity of vaginal secretion. American Journal of Obstetrics & Gynecology 172, 1268-1272.

Mai, T.L., Conner, D.E. 2007. Effect of temperature and growth media on the attachment of *Listeria monocytogenes* to stainless steel. International Journal of Food Microbiology 120, 282-286.

Marcone, V., Rocca, G., Lichtner, M., Calzolari, E. 2010. Long-term vaginal administration of *Lactobacillus rhamnosus* as a complementary approach to management of bacterial vaginosis. International Journal of Gynecology and Obstetrics 110, 223-226.

Mastromarino, P., Brigidi, P., Macchia, S., Maggi, L., Pirovano, F., Trinchieri, V., Conte, U., Matteuzzi, D. 2002. Characterization and selection of vaginal

Lactobacillus strains for the preparation of vaginal tablets. Journal of Applied Microbiology 93, 884-893.

Matu, M.N., Orinda, G.O., Njagi, E.N.M., Cohen, C.R., Bukusi, E.A. 2010. *In vitro* inhibitory activity of human vaginal lactobacilli against pathogenic bacteria associated with bacterial vaginosis in Kenyan women. Anaerobe 16, 210-215.

Mills, S., Stanton, C., Hill, C., Ross, R. 2011. New developments and applications of bacteriocins and peptides in foods. Annual Review of Food Science Technology 2, 299-329.

Moodley, P., Sturm, A.W. 2000. Sexually transmitted infections, adverse pregnancy outcome and neonatal infection. Seminars in Neonatology 5, 255-269.

Muench, D.F., Kuch, D.J., Wu, H., Begum, A.A., Veit, S.J., Pelletier, M.-E., Soler-García, Á.A., Jerse, A.E. 2009. H_2O_2-producing lactobacilli inhibit gonococci in vitro but not during experimental genital tract infection. Journal of Infectous Diseases 199, 1369-1378.

National Committee for Clinical Laboratory Standards, 2004. Approved Standard M7-A6. Performance Standards for Antimicrobial Susceptibility Testing, 14th ed., vol. 24(1) January.

Neto, M.T. 2008. Group B streptococcal disease in Portuguese infants younger than 90 days. Archives of Disease in Childhood – Fetal and Neonatal Edition 93, 90-93.

Nugent, R.P., Krohn, M.A., Hillier, S.L. 1991. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. Journal of Clinical Microbiology 29, 297-301.

O'Hanlon, D.E., Lanier, B.R., Moench, T.R., Cone, R.A. 2010. Cervicovaginal fluid and semen block the microbicidal activity of hydrogen peroxide produced by vaginal lactobacilli. BMC Infectious Diseases 10, 1-8.

Ocaña, V.S., Pesce de Ruiz Holgado, A.A., Nader-Macías, M.E. 1999. Characterization of a bacteriocin-like substance produced by a vaginal *Lactobacillus salivarius* strain. Applied and Environmental Microbiology 65, 5631-5635.

Owen, D.H., Katz, D.F. 1999. A vaginal fluid simulant. Contraception 59, 91-95.

Papagianni, M., Anastasiadou, S. 2009. Pediocins: the bacteriocins of Pediococci. Sources, production, properties and applications. Microbial Cell Factories 8:3.

Pascual, L.M., Daniele, M.B., Giordano, W., Pájaro, M.C., Barberis, I.L. 2008a. Purification and partial characterization of novel bacteriocin L23 produced by *Lactobacillus fermentum* L23. Current Microbiology 56, 397-402.

Pascual, L.M., Daniele, M.B., Ruiz, F., Giordano, W., Pájaro, C., Barberis, L. 2008b. *Lactobacillus rhamnosus* L60, a potential probiotic isolated from the human vagina. Journal of General and Applied Microbiology 54, 141-148.

Pavletic, A.J., Hawes, S.E., Geske, J.A., Bringe, K., Polack, S.H. 2004. Experience with routine vaginal pH testing in a family practice setting. Infectious Diseases in Obstetrics and Gynecology 12, 63-68.

Petricevic, L., Domig, K.J., Nierscher, F.J., Krondorfer, I., Janitschek, C., Kneifel, W., Kiss, H. 2012. Characterisation of the oral, vaginal and rectal *Lactobacillus* flora in healthy pregnant and postmenopausal women. European Journal of Obstetrics & Gynecology and Reproductive Biology 160, 93-99.

Pinto, A.L., Fernandes, M., Pinto, C., Albano, H., Castilho, F., Teixeira, P., Gibbs, P.A. 2009. Characterization of anti-*Listeria* bacteriocins isolated from shellfish: Potential antimicrobials to control non-fermented seafood. International Journal of Food Microbiology 129, 50-58.

Posfay-Barbe, K.M., Wald, E.R. 2009. Listeriosis. Seminars in Fetal and Neonatal Medicine 14, 228-233.

Puopolo, K.M. 2008. Epidemiology of neonatal early-onset sepsis. NeoReviews 9, 571-579.

Ramalheira, R., Almeida, M., Azeredo, J., Brandão, T.R.S., Almeida, G., Silva, J., Teixeira, P. 2010. Survival of clinical and food isolates of *Listeria monocytogenes* through simulated gastrointestinal tract conditions. Foodborne Pathogens and Disease 7, 121-128.

Rampersaud, R., Randis, T.M., Ratner, A.J. 2012. Microbiota of the upper and lower genital tract. Seminars in Fetal & Neonatal Medicine 17, 51-57.

Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M., Koenig, S.S.K., McCulle, S.L., Karlebach, S., Gorle, R., Russell, J., Tacket, C.O., Brotman, R.M., Davis, C.C., Ault, K., Peralta, L., Forney, L.J. 2011. Vaginal microbiome of reproductive-age women. Proceedings of the National Academy of Sciences of the United States of America 108, 4680-4687.

Reid, G. 2008. Probiotics and prebiotics – Progress and challenges. International Dairy Journal 18, 969-975.

Reid, G., Bocking, A. 2003. The potential for probiotics to prevent bacterial vaginosis and preterm labor. American Journal of Obstetrics & Gynecology 189, 1202-1208.

Reid, G., Kim, S.O., Köhler, G.A. 2006. Selecting, testing and understanding probiotic microorganisms. FEMS Immunology and Medical Microbiology 46, 149-157.

Reid, G., Soboh, F., Bruce, A.W., Mittelman, M. 1998. Effect of nutrient composition on the *in vitro* growth of urogenital lactobacilli and uropathogens. Canadian Journal of Microbiology 44, 866-871.

Reid, G., Burton, J., Hammond, J., Bruce, A.W. 2004. Nucleic acid-based diagnosis of bacterial vaginosis and improved management using probiotic lactobacilli. Journal of Medicinal Food 7, 223-228.

Riaz, S., Nawaz, S., Hasnain, S. 2010. Bacteriocins produced by *L. fermentum* and *L. acidophilus* can inhibit cephalosporin resistant *E. coli*. Brazilian Journal of Microbiology 41, 643-648.

Riley, M., Wertz, J. 2002. Bacteriocins: evolution, ecology, and application. Annual Review of Microbiology 56, 117-137.

Rinaudo, C.D., Rosini, R., Galeotti, C.L., Berti, F., Necchi, F., Reguzzi, V., Ghezzo, C., Telford, J.L., Grandi, G., Maione, D. 2010. Specific involvement of pilus type 2a in biofilm formation in group B *Streptococcus*. PLoS One 5. e9216.

Rojo-Bezares, B., Sáenz, Y., Poeta, P., Zarazaga, M., Ruiz-Larrea, F., Torres, C. 2006. Assessment of antibiotic susceptibility within lactic acid bacteria strains isolated from wine. International Journal of Food Microbiology 111, 234-240.

Romero, R., Schaundinn, C., Kusanovic, J.P., Gorur, A., Gotsch, F., Webster, P., Nhan-Chang, C., Erez, O., Kim, C.J., Espinoza, J., Gonçalves, L.F., Vaisbuch, E., Mazaki-Tovi, S., Hassan, S.S., Costerton, J.W. 2008. Detection of a microbial biofilm in intraamniotic infection. American Journal of Obstetrics & Gynecology 198, 135.e1-135.e5.

Ruiz-Moyano, S., Martin, A., Benito, M.J., Hernández, A., Casquete, R., Córdoba, M.G. 2011. Application of *Lactobacillus fermentum* HL57 and *Pediococcus acidilactici* SP979 as potential probiotics in the manufacture of traditional Iberian dry-fermented sausages. Food Microbiology 28, 839-847.

Schägger, H., Von Jagow, G. 1987. Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation of protein in the range from 1 to 100 kDa. Analytical Biochemistry 2, 368-379.

Schwebke, J.R. 2001. Role of vaginal flora as a barrier to HIV acquisition. Current Infectious Disease Reports 3, 152-155.

Semedo, T., Santos, M.A., Martins, P., Lopes, M.F.S., Marques, J.J.F., Tenreiro, R., Crespo, M.T.B. 2003. Comparative study using type strains and clinical and food isolates to examine hemolytic activity and occurrence of the cyl operon in enterococci. Journal of Clinical Microbiology 41, 2569-2576.

Simhan, H.N., Caritis, S.N., Krohn, M.A., Hillier, S.L. 2003. Elevated vaginal pH and neutrophils are associated strongly with early spontaneous preterm birth. American Journal of Obstetrics and Gynecology 189, 1150-1154.

Spelhaug, S.R., Harlander, S.K. 1989. Inhibition of food-borne pathogens by bacteriocins from *Lactococcus lactis* and *Pediococcus pentosaceus*. Journal of Food Protection 52, 856-862.

Spellerberg, B. 2000. Pathogenesis of neonatal *Streptococcus agalactiae* infections. Microbes and Infection 2, 1733-1742.

Stepanović, S., Ćirković, I., Ranin, L., Švabić-Vlahović, M. 2004. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Letters in Applied Microbiology 38, 428-432.

Stepanović, S., Vuković, D., Djukić, S., Ćirković, I., Švabić-Vlahović, M. 2007. Long-term analysis of *Listeria monocytogenes* vaginal carriage frequency in Belgrade, Serbia. Acta Microbiologica et Immunologica Hungarica 54, 195-199.

Stewart, P.S., Costerton, J.W. 2001. Antibiotic resistance of bacteria in biofilms. Lancet 358, 135-138.

Swaminathan, B., Gerner-Smidt, P. 2007. The epidemiology of human listeriosis. Microbes and Infection 9, 1236-1243.

Swenson, J.M., Facklam, R.R., Thornsberry, C. 1990. Antimicrobial susceptibility of vancomycin-resistant *Leuconostoc*, *Pediococcus* and *Lactobacillus* species. Antimicrobial Agents and Chemotherapy 34, 543-549.

Tamrakar, R., Yamada, T., Furuta, I., Cho, K., Morikawa, M., Yamada, H., Sakuragi, N., Minakami, H. 2007. Association between *Lactobacillus* species and bacterial vaginosis-related bacteria, and bacterial vaginosis scores in pregnant Japanese women. BMC Infectious Diseases 7, 128.

Tankovic, J., Leclercq, R., Duval, J. 1993. Antimicrobial susceptibility of *Pediococcus* spp. and genetic basis of macrolide resistance in *Pediococcus acidilactici* HM3020. Antimicrobial Agents and Chemotherapy 37, 789-792.

Tevi-Bénissan, C., Bélec, L., Lévy, M., Schneider-Fauveau, V., Mohamed, A.S., Hallouin, M.-C., Matta, M., Grésenguet, G. 1997. *In vivo* semen-associated pH neutralization of cervicovaginal secretions. Clinical and Diagnostic Laboratory Immunology 4, 367-374.

Tiago, I., Teixeira, I., Silva, S., Chung, P., Veríssimo, A., Manaia, C.M. 2004. Metabolic and genetic diversity of mesophilic and thermophilic bacteria isolated from composted municipal sludge on poly-epsilon-caprolactones. Current Microbiology 49, 407-414.

Todorov, S.D., Dicks, L.M.T. 2009. Bacteriocin production by *Pediococcus pentosaceus* isolated from marula (*Scerocarya birrea*). International Journal of Food Microbiology 132, 117-126.

Todorov, S.D., Botes, M., Danova, S.T., L.M.T., D. 2007. Probiotic properties of *Lactococcus lactis* ssp. *lactis* HV219, isolated from human vaginal secretions. Journal of Applied Microbiology 103, 629-639.

Tomás, M.S.J., Nader-Macías, M.E. 2007. Effect of a medium simulating vaginal fluid on the growth and expression of beneficial characteristics of potentially probiotic lactobacilli. Communicating Current Research and Educational Topics and Trends in Applied Microbiology 2, 732-739.

Turovskiy, Y., Ludescher, R.D., Aroutcheva, A.A., Faro, S., Chikindas, M.L. 2009. Lactocin 160, a bacteriocin produced by vaginal *Lactobacillus rhamnosus*, targets cytoplasmic membranes of the vaginal pathogen, *Gardnerella vaginalis*. Probiotics and Antimicrobial Proteins 1, 67-74.

Uehara, S., Monden, K., Nomoto, K., Seno, Y., Kariyama, R., Hiromi, K. 2006. A pilot study evaluating the safety and effectiveness of *Lactobacillus* vaginal suppositories in patients with recurrent urinary tract infection. International Journal of Antimicrobial Agents 28S, S30-S34.

Valore, E.V., Park, C.H., Igreti, S.L., Ganz, T. 2002. Antimicrobial components of vaginal fluid. American Journal of Obstetrics and Gynecology 187, 561-568.

van der Veen, S., Abee, T. 2011. Mixed species biofilms of *Listeria monocytogene*s and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid. International Journal of Food Microbiology 144, 421-431.

Van Reenen, C.A., Dicks, L.M.T., Chikindas, M.L. 1998. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. Journal of Applied Microbiology 84, 1131-1137.

Vera Pingitore, E., Bru, E., Nader-Macías, M.E. 2012. Effect of lyophilization and storage temperature on the activity of salivaricin CRL 1328, a potential bioactive

ingredient of a urogenital probiotic product. Journal of General and Applied Microbiology 58, 71-81.

Vera Pingitore, E., Hébert, E.M., Nader-Macías, M.E., Sesma, F. 2009. Characterization of salivaricin CRL 1328, a two-peptide bacteriocin produced by *Lactobacillus salivarius* CRL 1328 isolated from the human vagina. Research in Microbiology 160, 401-408.

Verani, J.R., McGee, L., Schrag, S.J. 2010. Prevention of perinatal group B streptococcal disease-revised guidelines from CDC, 2010. MMWR Recommendations and Reports 59, 1-32.

Vidhyasagar, V., Jeevaratnam, K. 2013. Evaluation of *Pediococcus pentosaceus* strains isolated from Idly batter for probiotic properties in vitro. Journal of Functional Foods 5, 235-243.

Vitali, B., Pugliese, C., Biagi, E., Candela, M., Turroni, S., Bellen, G., Donders, G.G.G., Brigidi, P. 2007. Dynamics of vaginal bacterial communities in women developing bacterial vaginosis, candidiasis, or no infection, analyzed by PCR-denaturing gradient gel electrophoresis and real-time PCR. Applied and Environmental Microbiology 73, 5731-5741.

Voravuthikunchai, S.P., Bilasoi, S., Supamala, O. 2006. Antagonistic activity against pathogenic bacteria by human vaginal lactobacilli. Anaerobe 12, 221-226.

Wasiela, M., Misiak, G., Pieczara, A., Kalinka, J. 2008. Association between hydrogen peroxide *Lactobacillus* species and bacterial vaginosis-related bacteria in vaginal fluid of pregnant women. Archives of Perinatal Medicine 14, 23-27.

Wilks, M., Wiggins, R., Whiley, A., Hennessy, E., Warwick, S., Porter, H., Corfield, A., Millar, M. 2004. Identification and H_2O_2 production of vaginal lactobacilli

from pregnant women at high risk of preterm birth and relation with outcome. Journal of Clinical Microbiology 42, 713-717.

World Health Organization, Food Safety Department. Modern food biotechnology, human health and development: an evidence based study 2005:9.

Yang, R., Johnson, M., Ray, B. 1992. Novel method to extract large amounts of bacteriocins from lactic acid bacteria. Applied and Environmental Microbiology 58, 3355-3359.

Zaragaza, M., Sáenz, Y., Portillo, A., Tenorio, C., Ruiz-Larrea, F., del Campo, R., Baquero, F., Torres, C. 1999. *In vitro* activities of ketolide HMR3647, macrolides, and other antibiotics against *Lactobacillus*, *Leuconostoc*, and *Pediococcus* isolates. Antimicrobial Agents and Chemoterapy 43, 3039-3041.

Zárate, G., Nader-Macias, M.E. 2006. Influence of probiotic vaginal lactobacilli on *in vitro* adhesion of urogenital pathogens to vaginal epithelial cells. Letters in Applied Microbiology 43, 174-180.

Zegels, G., Van Raemdonck, G.A., Coen, E.P., Tjalma, W.A., Van Ostade, X.W. 2009. Comprehensive proteomic analysis of human cervical-vaginal fluid using colposcopy samples. Proteome Science 7, 17.