Doctoral Thesis

Development and application of molecular tools for studying Bifidobacterium thermophilum RBL67 and Pediococcus acidilactici UVA1, two bacteriocin-producing human faecal co-isolates, in faeces and co-culture

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Development and application of real-time PCR tools for detection of *Bifidobacterium thermophilum* RBL67 and *Pediococcus acidilactici* UVA1, two bacteriocin-producing human faecal co-isolates

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Summary

Strains of the genus *Pediococcus* have a long history of use as natural preservatives in fermented food products, mainly due to their ability to produce pediocin, a broad spectrum antimicrobial peptide with strong antagonistic activity against *Listeria*. *Bifidobacterium* strains, on the other hand, are among the microorganisms being the most studied and used as probiotics in human because they are important components of the gastrointestinal flora and are usually regarded as safe. Although the underlying mechanisms by which specific bacteria function as probiotic organisms are far from understood, it is generally admitted that beside production of hydrogen peroxide and organic acids, bacteriocins or bacteriocin-like molecules play an important role.

Recently, two human faecal isolates, producing antimicrobial compounds of proteinaceous nature, were identified as *Pediococcus acidilactici* UVA1 and *Bifidobacterium thermophilum* RBL67. Based on the identity of these two strains, their close association, human origin and antimicrobial activities we formulated the hypothesis that they have potential for application as a probiotic mixed culture.

In the first part of this work, we identified the proteinaceous compound produced by *P. acidilactici* UVA1. The partially purified bacteriocin was heat resistant up to 100 °C, active over a wide range of pH (2 to 9), susceptible to proteolytic enzymes and had a molecular weight, estimated
by SDS-PAGE, of 4.5 kDa. Curing experiment, Southern hybridization and mRNA analysis showed that the antimicrobial activity was linked to the presence of the pediocin biosynthesis gene \textit{pedA}, which was plasmid-encoded. Nucleotide sequence of the whole operon (3.5 kb) showed more than 99.5 \% similarity to the pediocin AcH/PA-1 operon. Using a new real-time PCR assay, 11 out of 17 human faecal samples tested were found to contain \textit{pedA}-DNA.

In the second part of this thesis, oligonucleotides were designed for the development of specific PCR and real-time PCR assays for the detection of \textit{B. thermophilum} RBL67 in faeces. Specificity of the oligonucleotides was confirmed \textit{in silico} and \textit{in vivo} by PCR amplification on 17 \textit{Bifidobacterium} strains, representing 12 different species, and two lactobacilli. The real-time PCR assay showed a detection limit of $10^5$ cells of \textit{B. thermophilum} RBL67 per gram faeces. This test allowed to detect the presence of \textit{B. thermophilum} in one among 17 human faecal samples tested. Additionally, selective plating experiments led to purification of a faecal isolate of human origin, which 16S rDNA sequence was 99.93 \% similar to that of \textit{B. thermophilum} RBL67, confirming the presence of this species in the human gut.

Additionnaly, we found that \textit{B. thermophilum} RBL67 exhibited resistance to the antibiotic tetracycline and the resistance was attributed to the presence of the \textit{tet}(W) gene, which was detected on the genome of \textit{B. thermophilum} RBL67 by microarray hybridization and PCR amplification.

Furthermore, as first step towards identification of the bacteriocin thermophilicin B67, and because bacteriocin purification performed in another thesis was not successful, a molecular genetic method was started
for detection of the export signal sequences encoded on the genome of *B. thermophilum* RBL67; genomic libraries of translational fusions to the staphylococcal nuclease encoding gene devoid of its export signal were constructed in pFUN and established in *Escherichia coli*. In a next step, plasmids from the transformants will be transferred in *Lactococcus lactis* for screening of the nuclease activity and identification of export signal sequences present on the genome of *B. thermophilum* RBL67.

Finally, comparison of pure and mixed strain batch cultures of *P. acidilactici* UVA1 and *B. thermophilum* RBL67 showed a high stability of the co-culture, with both strains reaching high cell counts of $10^9$ cfu/ml after a very short incubation time of 4 hours for *P. acidilactici* UVA1 and 7 hours for *B. thermophilum* RBL67 and producing high anti-*Listeria* activity. Moreover, *B. thermophilum* RBL67 was not affected by the production of pediocin by *P. acidilactici* UVA1 during co-culture, showing potential for use as a probiotic mixture with antimicrobial properties. In addition, a gene-expression qPCR assay was successfully developed, used for the relative quantitation of the pediocin transcript *pedA* and demonstrated to be a valuable complementary tool to activity assay.
Resumé

L’utilisation des souches bactériennes du genre “Pediooccus” comme agents conservateurs naturels dans les produits alimentaires issus de la fermentation a une longue histoire. Ceci est principalement dû à la production de pédiocine, un agent antimicrobien à large spectre d’activité particulièrement actif contre Listeria. Les souches de bifidobactéries, quant à elles, sont parmi les organismes les plus étudiés et utilisés comme probiotiques, comptant parmi les composants majoritaires de la flore intestinale et étant considérées comme sans danger pour la consommation.

Malgré le fait que les mécanismes impliqués dans les propriétés probiotiques de certaines bactéries sont peu connus, il est généralement admis que, en plus de la production de peroxyde d’hydrogène et d’acides organiques, un rôle déterminant est joué par des protéines de faible masse moléculaire appelées bactériocines.

Récemment, deux bactéries isolées de matériel fécal humain et produisant des composés antibactériens de nature protéique ont été identifiées comme étant Pediococcus acidilactici UVA1 et Bifidobacterium thermophilum RBL67. Basés sur l’identité de ces souches, le fait qu’elle ont été isolées en tant que consortium, leur origine humaine et activités antimicrobiennes, nous avons proposé l’hypothèse selon laquelle P. acidilactici UVA1 et B. thermophilum RBL67 ont un potentiel comme culture mixte probiotique.
Dans la première partie de ce travail, nous avons identifié le composé protéique produit par *P. acidilactici* UVA1. La bactériocine partiellement purifiée est résistante à des températures allant jusqu’à 100 °C, active à des valeurs de pH comprises entre 2 et 9 et sensible au traitement par des enzymes protéolytiques. Sa masse moléculaire, estimée par SDS-PAGE, est de 4.5 kDa. Des expériences de “curing”, hybridation Southern et analyse des RNA messagers ont montré que l’activité antimicrobienne est liée à la présence du gène de biosynthèse de la pediocin, *pedA*, qui se trouve localisé sur un plasmide. La séquence nucléotidique de l’opéron entier est similaire à plus de 99.5 % à celui codant pour la pédiocine AcH/PA-1. L’utilisation d’un nouveau test de PCR en temps réel a permis la détection du gène *pedA* dans 7 des 11 échantillons fécaux testés.

Dans la seconde partie de cette thèse, des oligonucléotides ont été conçus pour le développement d’essais de PCR et PCR en temps réel pour la détection spécifique de *B. thermophilum* dans les fèces. La spécificité des oligonucléotides a été confirmée *in silico* et *in vivo* par amplification par PCR sur l’ADN de 17 bifidobactéries, représentant 12 espèces et de deux lactobacilles. La limite de détection de l’essai de PCR en temps réel a été déterminée à $10^5$ cellules de *B. thermophilum* RBL67 par gramme de fèces. Cette méthode a permis la détection de la présence de *B. thermophilum* dans un des 17 échantillons de matériel fécal humain testés. De plus, une expérience d’étalements sur agars sélectifs a permis d’isoler une souche de *B. thermophilum* d’origine humaine dont la séquence ADN codant pour l’ARN ribosomal 16S était à 99.93 % similaire à celle de RBL67. Ces résultats confirment la présence de *B. thermophilum* dans le système digestif humain.
De plus, nous avons observé que *B. thermophilum* RBL67 était résistante à l’antibiotique tétracycline et avons attribué cette résistance à la présence du gène *tet*(W), que nous avons détecté dans le génome de RBL67 par hybridation sur microarray et amplification par PCR.

Comme première étape d’identification de la bactériocine produite par *B. thermophilum* RBL67, puisque les tentatives de purification de la bactériocine pendant un précédent travail de thèse n’ont pas abouti, une méthode de génétique moléculaire a été appliquée pour la détection des séquences d’export présentes dans le génome de *B. thermophilum* RBL67. Une bibliothèque génomique de fusions traductionnelles entre l’ADN de RBL67 et le gène de la nucléase démuni de sa séquence d’export a été construite dans le plasmide pFUN et établie dans *Escherichia coli*. Les plasmides issus des transformants seront ensuite introduits dans *Lactococcus lactis* pour détecter l’activité de la nucléase et identifier des séquences d’export codées par *B. thermophilum* RBL67.

Finalement, la comparaison de cultures pures et mixtes de *P. acidilactici* UVA1 et *B. thermophilum* RBL67 a montré une grande stabilité de la co-culture. En effet, les deux bactéries atteignaient des comptes cellulaires élevés de $10^9$ cfu/ml après seulement 4 heures de culture pour *P. acidilactici* UVA1 et 7 heures pour *B. thermophilum* RBL67 et la culture mixte gardait un haut taux d’activité anti-*Listeria*. De plus, RBL67 n’était pas affectée par la production de pédiocine par UVA1 durant la culture mixte, indiquant un fort potentiel comme culture mixte probiotique avec propriétés antimicrobiennes. Un essai de PCR en temps réel a été développé et utilisé pour la mesure de l’expression du gène *pedA*. Cette méthode s’est avérée un outil précieux, complémentaire aux mesures phénotypiques d’activité anti-*Listeria*. 
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short chain fatty acid</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse phase HPLC</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>MALTI-TOF</td>
<td>Matrix-assisted laser desorption/ionization time-of-flight</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR = real-time PCR</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>rDNA</td>
<td>Ribosomal deoxyribonucleic acid</td>
</tr>
<tr>
<td>IS</td>
<td>Insertion sequence</td>
</tr>
<tr>
<td>NICE</td>
<td>Nisin-controlled gene expression system</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>CFS</td>
<td>Cell free supernatant</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent <em>in situ</em> hybridization</td>
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1 Introduction
1 Introduction

1.1 The gut microflora

With approximately 400 m², the gastrointestinal mucosa represents the largest surface of exchange with the environment within the human body and constitutes therefore a strong line of defense against foreign substances and microorganisms form the external environment.

Homeostasis of the individual with the external environment depends on the dynamic balance between the immune system, the mucosal barrier and the microflora (Guarner, 2006). The gut microflora is defined as the huge community of commensal bacteria colonizing the gastrointestinal mucosa; it plays an important role in the physiology of the host (Klijn et al., 2005). The interaction between gut bacteria and the host is a symbiotic relationship, where the host provides a nutrient-rich habitat and bacteria can bring important benefits on host’s health (Shanahan, 2002). The gut microflora is constituted of approximately ten fold more bacterial cells than human cells in the body, which represents 1-2 kg of bacteria within the adult gut (Shanahan, 2002; O’Hara and Shanahan, 2006).

Initially, the fetal gut is sterile, colonization begins immediately after birth and is influenced by the mode of delivery, infant diet, hygiene levels and medication (Grönlund et al., 1999). The gut microbiota is composed of hundreds of different bacterial species (Klijn et al., 2005), the most common genera represented being Bifidobacterium, Clostridium, Bacteroides, Eubacterium, Escherichia, Enterococcus, Streptococcus and Klebsiella (O’Hara and Shanahan, 2006).

The gut microflora has important and specific functions in both human health and disease. Evidences of these beneficial interactions were
reviewed by Guarner and Malagelada (2003), who suggested to group them in three categories: protective, structural and metabolic functions.

1.1.1 Protective function

The gut microbiota is considered to represent a crucial line of defense against colonization by exogenous or opportunistic bacteria that are present in the gut. This barrier effect probably involves several mechanisms, including displacement of pathogens by outcompeting them for nutrients and epithelial-binding sites (Guarner, 2006; Bernet et al., 1994), as well as production of antimicrobial factors, including lactic acid, $\text{H}_2\text{O}_2$ and bacteriocins that inhibit the growth of other bacteria (Shanahan, 2002).

1.1.2 Structural function

Comparison of germ-free mice with normally raised mice showed that gut bacteria play a role in barrier fortification by influencing the proliferation and differentiation of epithelial cells (Hooper et al., 2001; Falk et al., 1998) as well as the development of a competent immune system (Guarner and Malagelada, 2003).

1.1.3 Metabolic function

Gut bacteria also provide metabolic functions involved in host health. They have the ability to ferment non-digestible dietary substrates from the upper part of the gastrointestinal tract (GIT) and endogenous mucus produced by the epithelia. Fermentation of carbohydrates is a major source of energy in the colon for bacterial growth and produces short
chain fatty acids (SCFA) that can be absorbed by the host. This results in salvage of dietary energy contained in dietary fibers that would be lost otherwise (Blaut and Clavel, 2007) and favors the absorption of ions (Ca, Mg, Fe) in the caecum (Guarner, 2006). Bacteria in the gut have also been shown to be responsible for production of vitamins K, B\textsubscript{12}, biotin, folic acid and pantothenate, synthesis of amino acids from ammonia or urea (Hooper \textit{et al.}, 2002), inactivation of dietary carcinogens (Wollowski \textit{et al.}, 2001) and modulation of the immune system in a strain-dependent manner (Servin, 2004).

### 1.1.4 Negative effects on host health

Formation by the intestinal microflora of metabolites potentially deleterious to the host has also been reported. Mechanisms such as anaerobic metabolism of peptides and proteins, degradation of sulfur-containing amino acids or bile acids deconjugation lead to generation of potentially toxic or carcinogenic substances (Blaut and Clavel, 2007). Additionally, the gut microflora has been suggested to be an essential ingredient in the pathogenesis of Crohn’s disease and ulcerative colitis (Shanahan, 2002).

### 1.2 The probiotic approach

#### 1.2.1 Definition

The observations that the gut microflora can improve gastrointestinal function and protect humans against infection, that disturbance of this biota can increase susceptibility to infection and that ingestion of bacteria through diet could affect the composition of the microflora, led to the probiotic concept. At the beginning of the XX\textsuperscript{th} century, Metchnikoff
1.2 The probiotic approach

(1907) was the first to suggest the “replacement of harmful microbes by useful microbes” in the gut. Nowadays, probiotics are commonly defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002).

In addition to proven beneficial effects on the host, several criteria are generally proposed for the selection of microorganisms to be used as probiotic. These include human origin of the strain, safety aspects such as absence of transferable antibiotic resistance, acceptable sensory properties of the final food product, survival of the microorganism through the digestive tract and during large scale preparation and ability to efficiently colonize the GIT (Gibson and Fuller, 2000; Kolida et al., 2006).

1.2.2 Claimed effects of probiotics

Although only a small number of well-designed, double-blind clinical controlled trials are available to support the many health-promoting claims (Gionchetti et al., 2000), there are a variety of proposed beneficial health effects of probiotics (Rastall et al., 2005; Klaenhammer and Kullen, 1999; Marteau et al., 2001; Parvez et al., 2006) such as:

- Prevention of certain types of diarrhea
- Modulation of the immunity
- Strengthening of the barrier function or natural defenses in the gut by pathogen interference, exclusion and antagonism
- Maintenance of mucosal integrity
- Prevention or reduction of the effects in chronic intestinal inflammation
1 Introduction

- Improvement of lactose intolerance
- Decrease in the prevalence of allergy in susceptible individuals
- Reduction of serum cholesterol levels
- Anti-carcinogenic and anti-mutagenic activities

The most common way of administration of probiotics is through fermented dairy products (Leahy et al., 2005) since high bacterial cell counts (>10^6 bacteria per gram) are not allowed in non-fermented products in many countries and also because consumer’s acceptance is better, fermented dairy products like yoghurt being already associated with the consumption of bacteria.

Furthermore, the organisms being the most studied and used as probiotics in human are strains belonging to Lactobacillus spp. and Bifidobacterium spp. because they are considered important components of the gastrointestinal flora and are generally regarded as safe (Rolfe, 2000; Tuohy et al., 2005).

1.3 Bifidobacteria

1.3.1 Generalities

The genus Bifidobacterium includes the species of Bifidobacterium and Gardnerella and belongs to the family of Actinomycetaceae which also comprises corynebacteria, mycobacteria and streptomycoses (Klijn et al., 2005). Bifidobacteria are Gram-positive prokaryotes, non motile, non spore forming and non gas producing rods with genome sizes ranging from 1.9 to 2.9 Mb (Ventura et al., 2007). With a G+C content of 55-67 mol %, they belong to the high G+C content group. Being unable
to form colonies under aerobic conditions (20 % oxygen), they are classified as typical anaerobic bacteria. In liquid culture though, different species exhibit various degrees of sensitivity to oxygen. *B. boum* and *B. thermophilum* for example, showed growth stimulation under 20 % O₂ conditions compared to anoxic growth (Kawasaki *et al.*, 2006).

Bifidobacteria are saccharolytic organisms and all characterized strains have the ability to ferment glucose, galactose and fructose (Leahy *et al.*, 2005). Glucose is degraded exclusively and characteristically by the fructose-6-phosphate shunt. Fructose-6-phosphate phosphoketolase is the characteristic key enzyme of the bifid shunt that cleaves fructose-6-phosphate into acetyl phosphate and erythrose-4-phosphate (Meile *et al.*, 2001).

Bifidobacteria were first observed in 1899 after isolation from faeces of a breast-fed baby by Tissier (1899) who named them *Bacillus bifidus communis* because of their Y-shaped morphology. To date, 29 species are assigned to the genus *Bifidobacterium* (Felis and Dellaglio, 2007) which have been isolated mainly from the GIT of mammalians (Biavati and Mattarelli, 2005), but also from the GIT of honeybees, from the human vagina, human oral cavity and from sewage (Klijn *et al.*, 2005; Ventura *et al.*, 2004).

### 1.3.2 Bifidobacteria as member of the gut microflora

Bifidobacteria are dominant among the first colonizers of newborns and continue to persist at a low level in adults (Vaughan *et al.*, 2005). They are estimated to represent 40-50 % and 60-90 % of total bacteria in formula-fed and breast-fed infants, respectively (Harmsen *et al.*, 2000), but the population of bifidobacteria decrease after the weaning period and
they were shown to represent 3\% of the faecal flora in adults (Vaughan et al., 2002) until advanced age when the population of bifidobacteria appear to decline (Leahy et al., 2005).

As early colonizer and one of the dominant genera in the human gut, bifidobacteria are believed to play a pivotal role in maintenance of a healthy GIT (Klijn et al., 2005).

### 1.3.3 Probiotic potential of bifidobacteria

Their presence within the GIT has been associated with a number of health benefits such as combat diarrhea, relief of lactose intolerance, resistance to microbial infections, cancer prevention, treatment of inflammatory bowel disease, alleviation of constipation, immune function, and reduction of serum cholesterol (Leahy et al., 2005; Schell et al., 2002). Therefore, many attempts have been made to increase numbers of *Bifidobacterium* spp. in the intestinal tract by supplying certain bifidobacterial strains or food ingredients that stimulate the growth of bifidobacteria as food additives (Simmering and Blaut, 2001).

But to date, the underlying molecular mechanisms by which bifidobacteria function as probiotic organisms is far from understood (Leahy et al., 2005). It is generally admitted that production of antimicrobial substances such as hydrogen peroxide, organic acids, diacetyl, bacteriocins or bacteriocin-like molecules are implied in the beneficial health effects of bifidobacteria.
1.4 Pediococci

Pediococci are Gram-positive, homofermentative, facultatively anaerobic cocci belonging to the group of lactic acid bacteria. They usually occur in pairs or tetrads. In 2002, the genus *Pediococcus* was proposed to contain seven species: *Pediococcus damnosus*, *P. acidilactici*, *P. pentosaceus*, *P. parvulus*, *P. inopinatus*, *P. dextrinicus* and *P. claussenii* (Dobson et al., 2002). Since then, three new species were proposed: *P. cellicola* (Zhang et al., 2005), *P. stilesii* (Franz et al., 2006) and *P. ethanolidurans* (Liu et al., 2006). Some species exhibit extreme tolerances to temperature, pH and NaCl. For example, *P. acidilactici* grows at 50 °C whereas *P. damnosus* and *P. parvulus* are acid tolerant and grow at low temperatures (Stiles and Holzapfel, 1997). Although many single genes have been sequenced (comprising 16S rDNA and genes involved in production of the bacteriocin pediocin), only the complete genome of one strain of *Pediococcus pentosaceus* is available to date [GenBank: NC008525].

The pediococci were among the first bacteria to be described by Louis Pasteur in its “Etudes sur la Bière” (Pasteur, 1876). They are nowadays considered as the most prevalent spoilage microorganisms in the brewing industry, especially *P. damnosus* (Sakamoto and Konings, 2003). On the other hand, pediococci, particularly the strains *P. acidilactici* and *P. pentosaceus*, are of great economic importance in the food industry as starter cultures in fermented meat products, fermented vegetables, dough, fruit juices, dairy products as well as silage inoculants or probiotic feed products. Additionally, some *Pediococcus* strains are of particular importance in the food industry for use as biopreservation tools or probiotic strains through the production of the bacteriocin pediocin with
high anti-Listeria activity.

Strains belonging to the genus *Pediococcus* have been detected mainly in food products such as fermented cocoa (Kostinek *et al.*, 2008), bamboo shoots (Tamang *et al.*, 2008), in goat cheese (Bonetta *et al.*, 2008) and fermented dry sausage (Benito *et al.*, 2007). But there are also very recent reports of *Pediococcus* strains detected or isolated from human faecal samples (Millette *et al.*, 2007, 2008), porcine faeces or caeca (Casey *et al.*, 2004) and from canine intestine (Kim and Adachi, 2007).

### 1.5 Bacteriocins

#### 1.5.1 History and definition

Microorganisms produce a diverse and abundant array of toxic compounds as part of the intermicrobial competitive interactions. These substances include antibiotics, lactic acid, lytic agents, exotoxins and bacteriocins (Riley and Wertz, 2002). Many of these compounds have been adopted and adapted for the control of microbial populations (Kalmokoff *et al.*, 1996). Bacteriocin are the most abundant and diverse group of defense system (Riley and Gordon, 1999; Riley and Wertz, 2002) and have been defined by Cotter *et al.* (2005) as “gene-encoded, ribosomally synthesized antimicrobial, small, heat-stable peptides produced by one bacterium that are active against other bacteria, either in the same species (narrow spectrum), or across genera (broad spectrum) and to which the producer organism has an immunity mechanism”.

The first description of bacteriocin-mediated inhibition was reported more than 80 years ago, when antagonism between strains of *Escherichia coli* was first discovered (Gratia, 1925). Nowadays, it is speculated
that between 30-99 % of the Bacteria and Archaea make at least one bacteriocin (Klaenhammer, 1988; Riley, 1998). It is estimated that nearly 300 bacteriocins have been identified until now and the information on microbiological, structural and physicochemical properties of 123 of them were recently centralised in a web-accessible database, which should help gaining a better understanding of the biology of these peptides (Hamami et al., 2007).

Bacteriocins have found various fields of application; e.g. for plant disease control using bacteriocins that inhibit plant pathogenic bacteria (Montesinos, 2007), in human health for the control of bacterial infections or in the food industry for food -preservation or -safety as well as for the development of desirable flora in fermented food with bacteriocins produced by food-grade lactic acid bacteria (LAB) (Cotter et al., 2005).

1.5.2 Application in food products

Biopreservation can be defined as the addition of antagonistic cultures or of their metabolic products (organic acids, hydrogen peroxide, enzymes and bacteriocins) to food to inhibit pathogenic and spoilage bacteria and/or extend shelf life while changing the sensory properties of the product as little as possible (Castellano and Vignolo, 2006; Lücke, 2000; Holzapfel et al., 1995). The concept finds its origins in the growing consumers’ demands for foods that are ready to eat, fresh-tasting, nutrient and vitamin rich, but at the same time minimally-processed and preserved (Galvez et al., 2007).

Several groups of food-grade LAB produce bacteriocins which possess desirable properties that make them suitable for application in food products (Galvez et al., 2007);
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- being produced by food-grade bacteria, they are generally recognized as safe substances,
- absence of toxicity on eukaryotic cells,
- inactivated by the digestive proteases, they have little influence on the gut microbiota,
- they can endure harsh pH and temperature treatments, such as boiling, without loosing their activity (Nes and Johnsborg, 2004),
- their antimicrobial activity spectrum is broad, including many foodborne pathogenic and spoilage bacteria, for example strains of *Listeria*, *Clostridium* and other Gram-positive bacteria. Activity against Gram-negative bacteria has also been shown, but only in situations where the integrity of the outer membrane has been compromised (Cotter et al., 2005),
- they show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane and cross resistance with antibiotics are unknown,
- their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation.

There are basically two strategies for application of bacteriocin to food products: addition of the purified or partially purified bacteriocin in the product as food preservative or application of the bacteriocin-producing organism in food as starter, protective or probiotic culture. While bacteriocins added in food are likely degraded by the proteolytic enzymes of the stomach, consumption of bacteriocin-producing bacteria may lead to
in situ production of bacteriocins in GIT, where it can exert probiotic effect by inhibiting growth of pathogenic bacteria (de Vuyst and Leroy, 2007).

The effectiveness of bacteriocins preparation or bacteriocin producing microorganisms as food preservatives is well demonstrated and has been exploited for preservation of meat and dairy products, but also alcoholic beverages, egg products, fish, salad dressing and fermented vegetables (Cotter et al., 2005; Cleveland et al., 2001). To date, nisin is the only purified bacteriocin used commercially, but others, such as pediocin, also have found applications in food systems (Rodriguez et al., 2002).

**Nisin**

Nisin is a small (3.4 kDa, 34 amino acid), cationic, hydrophobic, lanthionine-containing peptide synthesized by *Lactococcus lactis* strains. It exhibits a wide activity spectrum, principally against Gram-positive bacteria whereas additions of chelating agents or detergents have been used to enhance its activity against Gram-negative bacteria. It was first marketed in England in 1953 and has since been approved for use in over 48 countries. As recently reviewed by Cotter et al. (2005), nisin was assessed to be safe for food use by the Joint Food and Agriculture Organization/World Heath Organization Expert Committee on Food Additives in 1969, was added to the European food additive list as number E234 in 1983 and was approved by the US Food and Drug Agency (FDA) for use in pasteurized, processed cheese spreads in 1988. Since then, it has been used as natural preservative in a variety of food products, mainly in dairy products (especially cheeses), canned goods (Chen and Hoover, 2003), liquid egg products, and salad dressings (Guder et al., 2000) for the control
of pathogenic bacteria, in particular the food borne pathogen *Listeria monocytogenes* or the spore-forming *Clostridium botulinum* (Ross et al., 2002).

**Pediocin**

Pediocin AcH/PA-1 is also a small (4.6 kDa, 44 amino acids) cationic peptide. Its range of antimicrobial activity is not as wide as for nisin (Millette *et al.*, 2007; Le Blay *et al.*, 2007), but pediocin displays a high specific activity against *Listeria monocytogenes*. This specific anti-*Listeria* activity has earned pediocin more attention by researchers to better characterize the bacteriocin because of the high mortality and morbidity rates associated with listeriosis (Kostrzynska and Bachand, 2006). Synthesis of pediocin PA-1 was initially detected in *Pediococcus acidilactici* strains (Gonzalez and Kunka, 1987; Bhunia *et al.*, 1987), but was also described in *P. pentosaceus*, *P. damnosus* and *P. parvulus* as well as in one *Lactobacillus plantarum* strain (Miller *et al.*, 2005; Green *et al.*, 1997). The bacteriocin received different names before realization that all were the same molecule (Rodriguez *et al.*, 2002).

The use of pediocin AcH/PA-1 for food biopreservation has been commercially exploited; the pediocin PA-1 containing fermentates Alta\(^\text{TM}\) 2341 (Quest International, Sarasota, FL) and Alta\(^\text{TM}\) 2351 are commercial food ingredients reported to extend shelf life of a variety of foods and, particularly, to inhibit the growth of *Listeria monocytogenes* in ready-to-eat meat products (Rodriguez *et al.*, 2002; Santiago-Silva *et al.*, In Press). Additionally, pediocin-producing *Pediococcus acidilactici* are used in the fermentation process of dry sausage production to improve food safety and preservation (Kostrzynska and Bachand, 2006).
1.5.3 Bacteriocin-producing bifidobacteria

Bacteriocin production by probiotic strains from different genera is thought to play an important role during *in vivo* interactions in the human GIT in combating gastrointestinal pathogenic bacteria.

Meghrous *et al.* (1990) were the first to show that bifidobacteria are able to produce antimicrobial substances against other Gram-positive species which had the properties of bacteriocins: proteinaceous nature, heat stable and active over a wide range of pH from 2 to 10. Four years later, Gibson and Wang (1994) reported the inhibition of *Escherichia coli* and *Clostridium perfringens* in co-culture with *Bifidobacterium infantis*, but it was not clear if the effect was related to bacteriocin or acid production. In 1998, Yildirim *et al.* described and purified a pediocin-like bacteriocin termed Bifidocin B and produced by *B. bifidum* NCFB 1454 (Yildirim and Johnson, 1998; Yildirim *et al.*, 1999). However, this strain was recently reclassified as *Pediococcus* sp. (unpublished data). Later on, Liévin *et al.* (2000) identified two bifidobacteria from human infant stool that expressed antagonistic activity against pathogens *in vitro*, inhibited cell entry, and killed intracellular *S. typhimurium* SL1344 in Caco-2 cells. The antibacterial components were found to be lipophilic molecules with a molecular weight of less than 3.5 kDa. More recently, Touré *et al.* (2003) isolated six bifidobacteria from infant faeces showing antimicrobial activity against *Listeria monocytogenes* that was due to proteinaceous compounds; they were able to partially purify the bacteriocins. Collado *et al.* (2005b,a) also isolated from human faeces six *Bifidobacterium* strains displaying wide spectrum antimicrobial activity against various Gram-positive and Gram-negative bacteria relevant to food safety and human health. They could link the ability to inhibit growth of *Helicobac-
ter *pylori* to heat-stable proteinaceous compounds (four with molecular weights above 10 kDa and two between 10 and 30 kDa).

However, none of these research groups did purify the inhibitory proteinaceous substances. Most of the compounds were partially purified by methanol-acetone extraction (Gibson and Wang, 1994; Touré *et al.*, 2003), ammonium sulfate precipitation, methanol-chloroform extraction or dialysis (Liévin *et al.*, 2000) or simply by filtration (Collado *et al.*, 2005b), but bacteriocin-like inhibitory compounds produced by bifidobacteria are likely difficult to purify, either because of their production in very low amounts or because they are atypical bacteriocins for which “classical” purification schemes are not applicable. As a result, no data is available yet on the genetic background of bacteriocin production by bifidobacteria.

### 1.5.4 Purification of bacteriocins

The effective use of bacteriocins in the food industry requires the understanding of their mode of action and genetic background, which in turn depends upon their purification and amino acid and nucleotide sequence determination. Absolute preliminary requirement is a sufficient yield of protein after purification (Carolissen-Mackay *et al.*, 1997). It is therefore important to optimize for each producing strain the culture conditions (media, fermentation conditions) as well as purification procedures, stability of the bacteriocin (the purer the peptide, the more unstable it becomes) and to start production studies with large batches.

The cationic and hydrophobic nature of bacteriocins is generally used for their recovery from complex fermentation broths which contain high levels of peptides (Parente and Ricciardi, 1999). Laboratory purification
protocols usually include an ammonium sulfate precipitation step, followed by various combinations of ion-exchange, hydrophobic interaction chromatography, gel filtration or chloroform / methanol extraction / precipitation with a final reversed-phase high-pressure liquid chromatography (RP-HPLC) step (de Vuyst and Leroy, 2007; Parente and Ricciardi, 1999). Using this “classical” purification scheme, bacteriocins such as curvacin A, sakacin P, lactosin S, bavaricin A, helveticin J, lactacin F and lactacin B were purified (Carolissen-Mackay et al., 1997). More recently, several protocols based on adsorption/desorption to producer cell in function of pH, expanded gel adsorption or phase partitioning have been developed (de Vuyst and Leroy, 2007; Parente and Ricciardi, 1999).

1.5.5 Purification of thermophilicin B67

Very recently, and as a part of this project, attempts were made to purify thermophilicin B67, the proteinaceous, antilisterial compound produced by *B. thermophilum* RBL67 (von Ah, 2006). This strain was previously shown to be moderately oxygen tolerant (12.5 % O₂ in reduced liquid medium) and to be able to grow in a large range of pH and temperature (von Ah, 2006). Furthermore, *B. thermophilum* RBL67 was shown in vitro to adhere to HT-29 and Caco-2 cell lines without being invasive and to block invasion of epithelial cells by *Listeria monocytogenes* (Moroni et al., 2006).

The antimicrobial compound produced by *B. thermophilum* RBL67 was shown to be heat-stable, active over a wide range of pH from 2 to 10 and sensitive to proteinase K, pronase E and trypsin. The crude preparation exhibited a narrow inhibition spectrum against *Lactobacillus acidophilus* DSM20079ᵀ, *Listeria ivanovii* HPB28, *Listeria innocua*
DSM20649 and all six *Listeria monocytogenes* strains tested.

Fermentation conditions were first screened to optimize bacteriocin production and the highest bacteriocin volumetric production (80 AU/ml·h) was obtained by repeated cycles batch culture with immobilized *B. thermophilum* RBL67 cells in semi-defined medium (Perrin *et al.*, 2001) at 40 °C and pH 6.0 (von Ah *et al*). Methanol-acetone extraction of the culture supernatant, being firstly described by Gibson and Wang (1994) as a suitable method for highly hydrophobic bacteriocins, resulted in a recovery of 35 % of the thermophilicin activity with a purification factor of 16.7. Further purification step by RP-HPLC resulted in total loss of measurable antimicrobial activity. Nevertheless, a protein of approximately 5 kDa could be detected in the methanol-acetone extract on silver-stained SDS-PAGE polyacrylamide gels and by MALDI-TOF analysis, possibly corresponding to thermophilicin B67. Given the unusual purification behavior of the bacteriocin, it was suggested that thermophilicin B67 presents a different structure than classical bacteriocins, making its purification even more challenging (von Ah, 2006).

### 1.6 Molecular tools for identification of unknown genes

Usual schemes for bacteriocin identification start with purification of the protein, but classical purification procedures seem to be inappropriate for bacteriocins produced by bifidobacteria. To circumvent these difficulties, molecular biology methods can be used to directly identify the gene(s) responsible for bacteriocin production.
1.6 Molecular tools for identification of unknown genes

1.6.1 Random mutagenesis

A widely used method for identification of unknown proteins is random mutagenesis of genomes followed by phenotypic screening of microorganisms for the absence of the searched trait. For identification of bacteriocin encoding genes, this could be a useful alternative, given that a mutation in one gene implied in synthesis, processing or transport of the protein would result in a negative signal when performing a rapid inhibition assay against a specific indicator organism.

Random mutagenesis with the help of genetic tools such as insertional elements (transposon or transposable plasmids) presents the advantage over chemical mutagenesis to allow localization of the insertion site and therefore direct identification of the interrupted gene. Transposition as a genetic tool has been abundantly described in the literature (Hayes, 2003). Among others, the following systems were developed and successfully used for genetic analyses of Gram-positive organisms.

**Tn916/Tn916\(\Delta E\)**

Tn916 is a 16.4-kbp transposon encoding tetracycline resistance and found originally in Gram-positive bacteria (Salyers et al., 1995). It has a broad host range and has been transferred successfully in many different microorganisms. Generally, the transposon, carried on a plasmid, is introduced into the target cells by electroporation. The vector being incapable of autonomous replication, survival of the target cells on medium containing antibiotic is dependant of successful integration of the transposon onto the chromosome. Tn916\(\Delta E\) is a variant of Tn916; it has been modified by replacement of the tetracycline resistance gene marker by an erythromycin resistance determinant. This change enlarges the range of
potential transfer recipient organisms, due to the relative large distribution of tetracycline resistance genes among microorganisms. Efficient mutagenesis by conjugal transfer of this transposon from *Enterococcus faecalis* RH110 (Rubens and Heggen, 1988) was shown among other in *Butyrivibrio fibrisolvens* strains (Hespell and Whitehead, 1991) and genes involved in pathogenesis of infective endocarditis caused by *Streptococcus mitis* (Bensing *et al.*, 2001), in cell wall teichoic acid glycosylation or flagellin expression by *Listeria monocytogenes* (Promadej *et al.*, 1999; Flanary *et al.*, 1999) were identified using this technique.

**pGH9:ISS1**

Maguin *et al.* (1996) developed a system based on the replication thermosensitive plasmid pGh+host as delivery vector, carrying the bacterial insertion sequence ISS1. Bacterial insertion sequences (IS) are a class of transposable elements which undergo replicate transposition leading to random integration of the plasmid vector between duplicated IS. The plasmid pGh9:ISS1 was shown to achieve high transformation efficiency in poorly transformable Gram-positive organisms and was widely used in lactococci, lactobacilli and streptococci for the identification of genes involved in defense or resistance mechanisms (Thibessard *et al.*, 2002; Boyd *et al.*, 2000; Ward *et al.*, 2001), in purine auxotrophy (Kilstrup and Martinussen, 1998), haemolytic activity (Spellerberg *et al.*, 1999), regulation of the phenolic acid metabolism (Gury *et al.*, 2004), phage adsorption (Dupont *et al.*, 2004) or for detection of a novel DNAse (Fontaine *et al.*, 2004).
1.6 Molecular tools for identification of unknown genes

Limitations

Prerequisites for application of random mutagenesis are first the availability of a simple and high-throughput screening method for rapid identification of the interesting mutants and on the other hand an efficient mean of delivery of the transposable element in the target organism, by electroporation or conjugation. Unfortunately, bacteriocins produced by bifidobacteria exhibit very low levels of inhibitory activity, which is usually not detectable by simple assays without concentration step. Moreover, there is no report in the scientific literature showing conjugal transfer into bifidobacteria and electroporation is known to be fastidious with these organisms.

1.6.2 Other molecular-based tools for LAB

To circumvent these difficulties, there also exists the possibility to conduct DNA experiments in more manipulation-friendly organisms such as *Escherichia coli* or *Lactococcus lactis*.

pFUN

The approach based on the plasmid pFUN consists in constructing genomic libraries of the target organism in an heterologous host and create fusions to a reporter gene for identification of protein export sequences. This kind of technique has a long history of use for identification of genes involved in protein secretion and the analysis of signal sequence structure and function (Payne and Jackson, 1991). The gene *LacZ*, encoding the beta-galactosidase has been extensively used for the investigation of protein excretion in *Escherichia coli* (Shuman and Silhavy, 2003).
1 Introduction

In 1985, Hoffman and Wright (1985) adapted this method by developing a reporter system based on the translational fusion to *phoA*, encoding an alkaline phosphatase. This protein was shown to be enzymatically active only when exported across the membrane making it an ideal tool for detection of protein export signals (Manoil et al., 1990).

Since then, the *phoA* reporter system has been adapted for application to a variety of Gram-negative bacteria by construction of a derivative of the Tn5 transposon containing the *phoA* reporter (Manoil and Beckwith, 1985) or placing the transposon onto broad-host range mobilizable plasmids (Taylor et al., 1989). With these adaptations, translational fusions to *phoA* were used to investigate the virulence genes in *Vibrio cholerae* (Taylor et al., 1989), to identify the export signal of pilin in *Pseudomonas aeruginosa* (Strom and Lory, 1987), the ferric enterobactin receptor FepA in *E. coli* K-12 (Murphy and Klebba, 1989) or to study protein secretion in such diverse microorganism as *Bacillus subtilis* (Payne and Jackson, 1991), *Streptococcus pneumoniae* (Pearce et al., 1993), *Mycoplasma fermentans* (Cleavinger et al., 1995), *Mycobacterium avium* (Carroll et al., 2000), *Staphylococcus aureus* (Williams et al., 2000) and *P. aeruginosa* PO1 (Lewenza et al., 2005).

Unfortunately, *E. coli*-derived PhoA is poorly active when expressed in Gram-positive hosts (Pearce et al., 1993). Therefore, adaptations of the system, well-developed for Gram-negative bacteria, were suggested by different groups for use in Gram-positive microorganisms. Gibson and Caparon (2002) developed a transposon containing an alkaline phosphatase gene (*phoZ*) derived from the Gram-positive bacterium *Enterococcus faecalis* as an export reporter. Poquet et al. (1998) used a different reporter: ΔspNuc, the *S. aureus* nuclease gene devoid of its export signal.
The staphylococcal nuclease is suitable for use as a reporter for signal peptides since the protein is only active extracellularly, its structure is simple and the codon usage in the gene is appropriate for high-level expression in lactococci (Ravn et al., 2000). With the method developed by Poquet and co-workers, exported proteins are detected after construction of a genomic library of the organism of interest in the plasmid pFUN. The plasmid contains a multiple cloning site upstream of the reporter gene devoid of signal sequence, in which heterologous DNA fragments can be cloned in the three reading frames for translational fusions and identification of export signals (Poquet et al., 1998). It has been used successfully by MacConaill et al. (2003) to investigate protein export in *B. breve* UCC2003. Similar approaches were used for protein secretion study in mycobacteria (Downing et al., 1999) and in *Lc. lactis* (Ravn et al., 2000).

**NICE**

The nisin controlled gene expression system (NICE) is also a widely used molecular tool developed more than 10 years ago for Gram-positive bacteria. It allows tightly controlled production of proteins from a variety of LAB and other industrially relevant bacteria, including *Bacillus*, *Enterococcus*, *Lactococcus*, *Lactobacillus* and *Streptococcus* spp. (de Vos, 1999). It was successfully used for diverse applications such as over-production of homologous or heterologous proteins, metabolic engineering, expression of membrane proteins, protein secretion, expression of genes with toxic products and large scale application (Mierau and Kleerebezem, 2005). Among others, the bacteriocins pediocin AcH/PA-1 from *Pediococcus acidilactici* and colicin from *Escherichia coli* were already heterologously
expressed using this system (Horn et al., 2004). The NICE system is very efficient for over-production of specific gene products for functional analyses, but is more likely to be used in a second step analysis as it requires that the gene to be analyzed be already identified.

1.6.3 Genome sequencing

The first Bifidobacterium genome sequence, that of B. longum NCC2705, was published in 2002 (Schell et al., 2002). Bioinformatic analysis of the complete sequence pointed out the high specialization of this strain to the colon environment: predicted proteins for the catabolism of a variety of carbohydrates, complete pathways for all amino acids and some vitamins and predicted proteins putatively involved in interaction with the host were revealed (Schell et al., 2002).

Later on, the B. breve UCC2003 genome sequence was also determined, but still not made publicly available (S. Leahy, M. O’Connell-Motherway, J. A. Moreno Munoz, D. Higgins, G. F. Fitzgerald, and D. van Sinderen, unpublished data). Genome sequencing of B. longum DJO10A is also complete, although not assembled yet and sequencing of the genomes of B. longum BORI and B. longum bv. infantis ATCC15697 are in progress.

Increasing availability of genomes of bifidobacteria in conjunction with development of more powerful and specific annotation tools will probably allow detection of bacteriocin genes on the genomes of bifidobacteria. The web server BAGEL (de Jong et al., 2006) for example, which combine information on conserved sequence motifs, genetic context of the putative bacteriocin encoding gene and a new up-to-date bacteriocin database, was recently developed for identification of putative bacteriocins open reading frames (ORF) that are often omitted during classical
annotation process, partly due to the small size and poor conservation of the bacteriocin genes. A query using this genome mining tool with the genomic sequence of *B. longum* (1798 coding sequences) gave 3 significant results: one locus showing similarity to holin-like protein, and two “hypothetical proteins” which would merit further analysis.

1.7 Real-time PCR

1.7.1 Principle

PCR (polymerase chain reaction) is a laboratory routine technique aimed to specific amplification of DNA fragments under the action of a DNA polymerase. Theoretically, the target-DNA molecule is replicated after each PCR cycle. Following this reasoning, and under optimal conditions, after n cycles, the PCR mixture should contain $2^n$ fold more target DNA than was present at the beginning of the reaction. But in reality (Figure 1.1), optimal conditions only occur in a limited period (exponential amplification phase). At a certain point during the amplification, reagents start to deplete and reaction slows down (linear phase). During the plateau phase, amplification stops and PCR products even start to degrade. Reliable quantitative data can only be collected during exponential amplification, when amount of detected DNA is truly proportional to the initial amount of starting target material.
This is precisely the principle of the real-time PCR (or quantitative PCR or qPCR): amplification of the target DNA is monitored in real-time by the help of a specific fluorescent reporter dye. The reporter dye emits a fluorescent signal whose intensity is proportional to the amount of target DNA present after each amplification cycle. The number of cycles after which fluorescent intensity rises above background noise is called the $C_T$ value (Figure 1.2). It is measured for each sample and is inversely proportional to the initial copy number of target DNA, allowing accurate quantification. Samples that differ by a factor of 10 in the original concentration of DNA, for example, would be $\sim 3.3$ cycles apart ($\Delta C_T = 3.3$).
Figure 1.2: Amplification curves of two DNA samples by real-time PCR. The intensity of the emitted fluorescence is measured after each cycle. The $C_T$ value is defined as the number of cycles after which the emitted fluorescence rises above background (crosses the threshold line).

Advantages of this technique are high throughput, high specificity and sensitivity and the absence of requirement of any post-PCR manipulation (Bustin et al., 2005). Disadvantages are the high sensitivity to contamination, inaccuracies due to differences in DNA extraction or reverse transcription efficiencies, biases introduced by inadequate normalization and the high cost of the material and consumables (Nolan et al., 2006).
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1.7.2 Applications

Real-time PCR is considered a superior method for direct detection and quantification of larger bacterial groups in complex environments (Rinttila et al., 2004). The method has been used by many authors to study the distribution of various bacterial populations in the intestinal microbiota to gain better knowledge about the GIT community composition (Rinttila et al., 2004; Hopkins et al., 2005; Requena et al., 2002; Gueimonde et al., 2004; Matsuki et al., 2004). More specifically, the use of group specific real-time PCR has been described for the assessment of efficacy of probiotic and prebiotic and the changes they induce in the microbiota (de Preter et al., 2008; Carey et al., 2007) or for determination of the influence of diet or disease on the gut microflora composition (Firmesse et al., 2007; Malinen et al., 2005).

Real-time PCR is also widely used for detection and/or quantification of single bacterial species or specific genes in various samples. This has been applied in such various areas as detection of pathogens microorganisms in food products (Yang et al., 2007), clinical diagnosis (Peterson et al., 2007; Bélanger et al., 2003), control of wastewater treatments (Wéry et al., 2008) or evaluation of the persistence of a probiotic strain after administration (Horz et al., 2007).

Coupled to reverse transcription, real-time quantitative PCR is also used as a rapid and reliable tool for determination of bacterial cell viability. This application is based on the observation that turnover of mRNA is high in living, metabolically active cells, making it a good indicator of cell viability (Sheridan et al., 1998). Aellen et al. (2006) validated use of quantification of a 16S rRNA fragment in medical microbiology for determination of drug-induced killing of Streptococcus gordonii.
1.8 Aim of the thesis

For transcriptional analysis of bacteriocin production, real-time PCR coupled with reverse transcription is also a method of choice, allowing quantification of a specific mRNA, and therefore of the expression of a bacteriocin without need for a phenotypic activity assay. Gene expression real-time PCR assays were recently used for the study of the in vitro expression of the bacteriocins produced by a *Lactobacillus sakei* strain (Vaughan *et al.*, 2004) and a strain of *L. plantarum* (Ramiah *et al.*, 2007).

1.8 Aim of the thesis

1.8.1 Background

In a study aimed to characterize new *Bifidobacterium* isolates of human origin, Touré *et al.* (2003) isolated 34 bifidobacteria from infant faeces. Six isolates showed high antagonistic activity against *L. monocytogenes*. The activities were heat-stable but sensitive to proteinase treatment, indicating a proteinaceous character. After microscopic observation and PCR analysis, one isolate was shown to consist of a consortium of two strains (von Ah, 2006). The strains were identified as *Pediococcus acidilactici* and *Bifidobacterium thermophilum* and were named UVA1 and RBL67, respectively (von Ah *et al.*, 2007). It was demonstrated that both strains participated in the antimicrobial activity by production of proteinaceous compounds (von Ah *et al.*, 2007).

1.8.2 Hypothesis

*P. acidilactici* UVA1 and *B. thermophilum* RBL67, two bacteriocin producing human faecal isolates, have potential for application as a probiotic mixed culture with antimicrobial features.
1 Introduction

1.8.3 General objective

The general objective of this work is to develop and apply molecular genetic tools for the characterization of bacteriocins produced by the two strains *P. acidilactici* UVA1 and *B. thermophilum* RBL67 and to study strains interactions and anti-Listeria activity in mixed cultures. Furthermore, the distribution of these two strains in human faecal samples was investigated.

1.8.4 Specific objectives

The specific objectives of this work are:

- To identify the bacteriocin produced by *P. acidilactici* UVA1.
- To identify and characterize the genetic background of the bacteriocin produced by *B. thermophilum* RBL67.
- To study the distribution of pediocin-producing strains and *B. thermophilum* in human faeces.
- To characterize the interactions of the two strains in a co-culture system.
- To study bacteriocin production during pure and co-cultures.
2 Detection of the pediocin gene *pedA* in strains from human faeces by real-time PCR and characterization of the bacteriocin produced by *Pediococcus acidilactici* UVA1

Data presented in this chapter were published in 2007 in BMC Biotechnology (Mathys et al., 2007)
2.1 Abstract

2.1.1 Background

Bacteriocin-producing lactic acid bacteria are commonly used as natural protective cultures. Among them, strains of the genus *Pediococcus* are particularly interesting for their ability to produce pediocin, a broad spectrum antimicrobial peptide with a strong antagonistic activity against the food-borne pathogen *Listeria monocytogenes*. Furthermore, there is increasing interest in isolating new bacteriocin-producing strains of human intestinal origin that could be used for probiotic effects and inhibition of pathogenic bacteria in the gut. In this work, we characterized the proteinaceous compound with strong antilisterial activity produced by *Pediococcus acidilactici* UVA1, a human baby faeces isolate.

2.1.2 Results

Strain UVA1 was previously identified as a *Pediococcus acidilactici* by carbohydrate fermentation profile, growth at 50 °C and 16S rDNA sequencing. The partially purified bacteriocin was heat resistant up to 100 °C, active over a wide range of pH (2 to 9) and susceptible to proteolytic enzymes. The molecular weight, estimated by SDS-PAGE, was similar to that of pediocin AcH/PA-1 (4.5 kDa). *P. acidilactici* UVA1 harboured a 9.5-kb plasmid that could be cured easily, which resulted in the loss of antimicrobial activity. Southern hybridization using the DIG-labelled *pedA*-probe established that the bacteriocin gene was plasmid-borne as for all pediocin described so far. Nucleotide sequence of the whole operon (3.5 kb) showed almost 100 % similarity to the pediocin AcH/PA-1 operon. The mRNA transcript for *pedA* could be detected
in *P. acidilactici* UVA1 but not in the cured derivative, confirming the expression of the *pedA*-gene in UVA1. Using a new real-time PCR assay, eleven out of seventeen human faecal samples tested were found to contain *pedA*-DNA.

2.1.3 Conclusion

We identified and characterized the first pediocin produced by a human intestinal *Pediococcus acidilactici* isolate and successfully developed a new real-time PCR assay to show the large distribution of *pedA*-containing strains in baby faecal samples.

2.2 Background

The lactic acid bacteria are an inhomogeneous group which includes among others the genera *Pediococcus*, *Enterococcus*, *Lactococcus*, *Lactobacillus* and *Streptococcus*. Among the *Pediococcus* genus, *P. acidilactici* and *P. pentosaceus* are widely used for fermentation of foods like vegetable and meat products. By production of organic acids, resulting in pH decrease, they contribute to control the microbial succession during fermentation. They also act as protective cultures preventing the growth of food-borne pathogens such as *Listeria monocytogenes* or *Staphylococcus aureus* (Drider *et al.*, 2006) and by doing this, they extend storage life and enhance safety of food products (Devlieghere *et al.*, 2004; Cotter *et al.*, 2005). Beside production of classical antimicrobial compounds such as organic acids or hydrogen peroxide, the protective effect also results from the production of bacteriocins (Eijsink *et al.*, 2002).

Bacteriocins are ribosomally synthesized, small, heat-stable antimicro-
bacterial peptides produced by bacteria. Pediocin AcH/PA-1 is the most studied class IIa bacteriocin (non modified, nonlantibiotic peptides) and has potential for use as food preservative, due to its strong anti-
Listeria activity (Rodriguez et al., 2002). Since amino-acid sequence determination of the pediocin AcH/PA-1 in 1992 (Henderson et al., 1992; Ni
eto Lozano et al., 1992), several pediocin-producing P. acidilactici and
P. pentosaceus strains have been screened from a large variety of plants and fruits (Carr et al., 2002), but also from the gastrointestinal tract of poultry, ducks and other animals (Juven et al., 1991; Kurzak et al., 1998; Hudson et al., 2000). Millette et al. (2007) recently isolated a strain of Pe
diococcus acidilactici from human faeces which produces an unidentified antimicrobial proteinaceous compound. To our knowledge, no pediocin-
producing Pediococcus has been isolated so far from human faeces and it is therefore unknown to which extent these strains are widespread in the human intestinal microbiota and contribute to the microbial balance of the complex gut ecosystem.

Real-time PCR is a very sensitive and rapid molecular method for the detection of specific genes in complex samples. It is particularly suitable for measuring non cultivable bacteria, because detection is independent of growth conditions of the target organism (Stewart et al., 2006). It has for example recently been used successfully on faecal samples to assess the survival of Lactococcus lactis subsp. cremoris FC after transit through the gastrointestinal tract (Maruo et al., 2006) or to detect the presence of noroviruses in clinical stool samples (Houde et al., 2006).

In this work, we report the biochemical and genetic characterization of the antimicrobial compound produced by the new bacteriocin-producing strain Pediococcus acidilactici UVA1, which was co-isolated from baby
faeces with the bacteriocinogenic strain *Bifidobacterium thermophilum* RBL67 (Touré *et al.*, 2003). The distribution of *pedA*-containing strains in human faeces was also investigated using a newly designed real-time PCR assay targeting the pediocin structural gene.

### 2.3 Material and methods

#### 2.3.1 Bacterial strains, growth conditions and plasmid-curing

*Lactobacillus paracasei* subsp. *paracasei* DSM5622<sup>T</sup>, *Pediococcus acidilactici* DSM20284<sup>T</sup> and *Pediococcus pentosaceus* DSM20336<sup>T</sup> (obtained from DSMZ GmbH, Braunschweig, Germany) were used as reference strains for analysis of the carbohydrate fermentation profile. *Listeria ivanovii* HPB28 (obtained from the Health Protection Branch, Health and Welfare, Ottawa, Canada) was used as indicator strain for the detection of pediocin activity (Daba *et al.*, 1994).

*P. acidilactici* UL5 (own culture collection) was used as pediocin producer control (Daba *et al.*, 1994). *Pediococcus acidilactici* UVA1 was previously isolated from human baby faeces as a stable consortium with *B. thermophilum* RBL67 (Touré *et al.*, 2003; von Ah *et al.*, 2007). Strain UVA1 was purified by subsequent selective plating and analysis of single colonies by genus-specific probes (von Ah, unpublished). A non-bacteriocin producing derivative of *P. acidilactici* UVA1, named UVA1-bac<sup>−</sup>, was obtained after curing by novobiocin treatment (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland, 0.1 µg/ml) as already described (Halamí *et al.*, 2000). Sixty colonies were screened by the overlay method for absence of antimicrobial activity against *L. ivanovii* HPB28 and four mutants producing no inhibition halo were detected. One of them was
purified on MRSC agar, consisting of MRS (de Man et al. (1960), Biolife, Milan, Italy) supplemented with 0.05 % L-cysteine hydrochloride and 1.5 % agar, and propagated in MRSC broth. The cell-free supernatant was checked for the absence of anti-Listeria activity and absence of the pedA-gene was confirmed by PCR.

All lactic acid bacteria were routinely grown overnight in MRSC medium, with incubation at 37 °C in anaerobic jars with an atmosphere generation system (Oxoid AnaeroGen TM, Basel, Switzerland). *L. ivanovii* HPB28 was propagated in TSY medium consisting of tryptic soy broth (Oxoid) containing 0.6 % (w/v) yeast extract (Merck, Darmstadt, Germany) overnight at 30 °C. For agar plates and soft-agar, the media were supplemented with 1.5 % and 0.75 % (w/v) agar, respectively. Bacterial stocks were stored at -80 °C in appropriate media supplemented with 33 % (v/v) glycerol and subcultured three times at one day intervals on fresh agar plates before use.

### 2.3.2 Inhibition assay

Antibacterial activity was assessed by the agar-well diffusion method. Briefly, 25 ml of soft-agar (heated at 45 °C) was inoculated with 0.1 % of an overnight culture of the indicator strain *L. ivanovii* HBP28, poured into a Petri dish and allowed to set for 30 min at room temperature. Holes (diameter of 7 mm) were then punched in the agar and filled with 80 µl of sample. The plates were incubated at 4 °C for 30 min to allow bacteriocin diffusion and overnight at 30 °C for growth of the indicator strain. The diameter of the inhibition zone was measured.
2.3 Material and methods

2.3.3 Effect of temperature, pH, enzymes and other agents on bacteriocin activity

Cell-free supernatant (CFS) was obtained after centrifugation at 13’000 x g for 10 min at 4 °C of a 16-h culture in MRSC at pH 6 and 37 °C. The supernatant was heated 5 min at 95 °C. The effect of temperature on the antibacterial activity was tested after heating at 121 °C for 15 min in an autoclave and at 100 °C for 60 and 40 min using a water-bath. The effect of pH was tested by adjusting the pH of the CFS to values in a range from 2 to 11 using either 1 M HCl or 1 M NaOH. Residual activity was measured by the agar-well diffusion method, after one day, one week and one month storage at 4 °C. To test the sensitivity to proteases and other agents, the CFS was incubated for 2 h at 37 °C in the presence of 1 mg/ml chymotrypsin, pepsin, protease, proteinase K, trypsin or lysozyme or 1 % SDS, urea, catalase, RNAse A, Tween 20, Tween 80, Triton-X or 2, 5 or 10 mM EDTA. For enzyme denaturation, the samples were finally heated at 95 °C for 5 min and residual activity was measured by the agar-well diffusion method. All enzymes and other chemicals were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland), except proteinase K and trypsin, which were obtained from Applichem (Darmstadt, Germany). Residual activity was defined as the ratio of the diameter of the halo produced by the treated sample compared to the untreated control and expressed in percentage. All assays were performed twice.

2.3.4 Molecular weight determination

The bacteriocin produced by P. acidilactici UVA1 was partially purified by injecting 300 ml CFS at a rate of 1 ml/min in a 60-ml SP Sepharose
column connected to a FPLC chromatography system (Ätka Purifier 10, Amersham, Otelfingen, Switzerland). The column was first equilibrated with 10 column volumes of 5 mM ammonium acetate buffer (pH 5.0), 5-ml fractions were collected in a fraction collector (Frac-950, Amersham) and the bacteriocin-like activity was eluted with 0.45 M NaCl in the same buffer.

The CFS and active fractions after FPLC were 10-fold concentrated by ultrafiltration (cutoff of 3 kDa) and 15 µl of the samples were loaded on two parallel SDS gels, along with 10 µl of polypeptide SDS-PAGE molecular weight standard (BioRad Laboratories AG, Reinach, Switzerland). The gels were prepared according to Schägger and von Jagow (1987) and consisted of a 10 % acrylamide-bisacrylamide stacking gel and a 16.5 % separating gel. Separation was done with constant voltage (100 V) for 2.5 h using a vertical slab gel apparatus (BioRad Laboratories). One of the gels was stained with Coomassie brilliant blue R250 (LK Bromma, Villeneuve-la-Garenne, France) and the other was used for activity detection: the gel was first soaked for 2 h in fixation solution (20 % isopropanol, 10 % acetic acid) and rinsed overnight in HPLC-grade water before being overlaid with 25 ml soft TSY agar inoculated with 0.1 % of an overnight culture of *L. ivanovii* HBP28. The molecular weight was estimated by comparison of the mobility of the inhibition zone to that of the molecular weight marker run simultaneously. The whole procedure was repeated twice.

2.3.5 DNA sequencing and PCR conditions

Sequencing of DNA was performed by Microsynth (Balgach, Switzerland) and similarity searches were conducted with the BLAST program from
2.3 Material and methods

NCBI (version 2.2.15). Primers and probe used in this study are listed in Table A.1. They were designed with the program Primer3 (Rozen and Skaletsky, 2000) and synthesised by Microsynth. The PCR reactions were set up in a total volume of 50 µl containing 2.5 U EuroTaq-DNA-Polymerase (Digitana, Horgen, Switzerland), 1.5 mM MgCl₂ (Digitana), 0.2 mM dNTP’s (GE Healthcare), 0.5 µM of each primer and either 2 µl of DNA or 40 µl of cell suspension (prepared by resuspending a single colony in 210 µl of sterile, double distilled water).

For amplification and sequencing of the first 711 bp of the pediocin PA-1 operon, primers P1 and P2 and conditions described by Rodriguez et al. (1997) were used. The second part of the operon (2864 bp) was amplified with primers pedopF and pedopR, designed on the basis of the reported sequence for pSRQ11 (Marugg et al., 1992). Amplification conditions were as follow: 2 min at 95 °C, 30 cycles of 1 min at 94 °C, 35 s at 45 °C and 3 min at 72 °C and final elongation step 7 min at 72 °C. Oligonucleotides pedopF and pedopR as well as pedseq A, B, C and D, designed every 500 bp along the PCR product, were used as sequencing primers. Additionally, a 1009-bp sequence directly upstream and a 1417-bp sequence directly downstream of the operon were amplified and sequenced with primer pairs pedseq L and H and pedseq M and N, respectively. Amplification conditions were: 3 min at 95 °C, 30 cycles of 1 min at 95 °C, 35 s at 55 °C and 2 min at 72 °C and final elongation step 7 min at 72 °C.

2.3.6 Plasmid isolation and Southern blotting

Extrachromosomal DNA elements were extracted from *P. acidilactici* UVA1, UL5 and UVA1-bac⁻ using a modified method after Anderson and
McKay for small scale plasmid isolation (Anderson and McKay, 1983). Shortly, for cell lysis, 9.5 µl mutanolysin (1500 U/ml, Sigma-Aldrich Chemie GmbH) was added to the lysis solution (solution B) and plasmid DNA was resuspended in 1 x TE buffer. Finally, the RNA was digested with 10 µg RNase A (Sigma-Aldrich Chemie GmbH).

The DNA was visualized after electrophoresis on a 0.65 % agarose gel in 1 x TBE at 100 V for 1.5 h. The supercoiled DNA ladder (Promega, Madison WI, USA) was used as size standard. DIG-labelling of the pedA-probe (P1-P2 PCR product on P. acidilactici UVA1), blotting on nylon membrane, hybridisation (at 42 °C) and chemiluminescent detection were conducted with the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics, Rotkreuz, Switzerland) according to supplier’s instructions. Plasmid preparation, blotting and hybridization were performed twice.

2.3.7 RNA isolation and reverse transcription

The RNA was isolated during the exponential growth-phase of P. acidilactici UVA1, UVA1-bac−, UL5 and DSM20284T, and P. pentosaceus DSM20336T using the RNeasy Mini kit (Qiagen, Basel, Switzerland). The protocol was slightly modified by addition of 20 U mutanolysin in the lysis solution. The samples were finally treated with RNase-free DNAse I (Invitrogen, Basel, Switzerland) for 30 min at 37 °C.

First strand cDNA synthesis was performed with the Omniscript reverse transcription kit (Qiagen) and 5 µl of the product were used for PCR amplification of a 100 bp-fragment with primers pedA2RTF and pedA2RTR. PCR products were separated on a 2 % agarose gel in 1 x TAE buffer by electrophoresis at 90 V for 2.5 h. The low molecular weight DNA
2.3 Material and methods

Ladder and Tridye 100 bp DNA-ladder (New England BioLabs, Ipswich, MA, USA) were used as size standards.

2.3.8 Preparation of DNA from faecal samples

Twenty-one human faecal samples were collected in collaboration with the Department of Gastroenterology (Hospital for Sick Children, Zurich, Switzerland). Thirteen faecal samples were collected from children donors aged one month to 3 years and four from adults. Faecal samples were collected within 1 h after defecation, placed in anaerobic jars and rapidly transported to our laboratory. They were immediately frozen at -20 °C upon arrival, i.e. no more than 3 h after defecation.

Total DNA was isolated from 200 mg of each sample using the QIAamp DNA Stool Mini kit (Qiagen) according to the manufacturer’s instructions. Before DNA extraction, one faecal sample was autoclaved twice (121 °C, 15 min) to obtain a PCR-amplification negative sample. Ten aliquots of this sample were spiked with a 10-fold serial dilution of *P. acidilactici* UVA1 (overnight culture in MRSC) at concentrations ranging from $10^9$ to $10^1$ bacteria cells per g faeces. The extracted DNA was stored at -20 °C.

2.3.9 Real-time PCR

Sequences of primers pedA2RTF and pedA2RTR as well as TaqMan probe TqMpedA are reported in Table A.1. They were designed for specific amplification of a 100-bp fragment based on the *pedA* sequence with the software PrimerExpress 1.5 (Applied Biosystems, Rotkreuz, Switzerland) and synthesized by Microsynth. The TaqMan probe was labeled
with 5’-FAM as a fluorescent reporter dye and 3’-TAMRA as a quencher. Their specificity was tested using the BLAST program from NCBI.

Reactions were set in a total volume of 25 µl, containing 2.5 µl of faecal DNA extract, 12.5 µl of qPCR MasterMix from Eurogentec (Seraing, Belgium), 0.3 µM of each primer and 0.1 µM of the TaqMan probe. Reactions were run on an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Rotkreuz, Switzerland). The amplification conditions were 2 min at 50 °C, 10 min denaturation at 95 °C, followed by 45 cycles of 15 sec at 95 °C and 1 min at 60 °C. The cycle threshold (C$_T$), corresponding to the number of cycle after which the target-DNA concentration increase become exponential, was monitored. Results were analysed using the SDS 2.1 Software (Applied Biosystems). All reactions were done in triplicate and repeated three times.

2.4 Results

2.4.1 Biochemical characterization of the antimicrobial compound produced by *P. acidilactici* UVA1

Heating the cell-free supernatant (CFS) at 121 °C for 15 min destroyed the inhibitory activity against *Listeria ivanovii* HPB28, whereas heat treatments at 100 °C for 40 and 60 min only yielded a slight reduction of the activity with 80 and 75 % residual activity, respectively. Activity
remained unaffected after one month storage at 4 °C at pH values from 2 to 8. At pH 10 and 11, total loss of activity was already observed after 1 week storage and only 60 % of the initial activity was still measurable after 1 month at pH 9. Treatment with proteolytic enzymes such as chymotrypsin, pepsin, protease, proteinase K or trypsin resulted in a total loss of activity while lysozyme, catalase and other agents tested had no effect.

2.4.2 Determination of the molecular weight

SDS-PAGE of the partially purified bacteriocin (concentrated active FPLC-fraction) showed a diffuse band on the Coomassie-stained gel while a clear inhibition halo was visible on the activity-gel for the concentrated FPLC-fraction as well as for the CFS (Figure 2.1). The relative mobility of the inhibition zone on the gel overlaid with *Listeria ivanovii* HBP28 was compared to that of the standards stained with Coomassie blue. The apparent molecular weight of the bacteriocin was estimated in the range of 4.5 to 5 kDa. The biochemical properties and the molecular weight described above, as well as the fact that this proteinaceous compound is produced by a *Pediococcus* strain suggest that the anti-*Listeria* compound is a pediocin-like bacteriocin. A genetic approach was used to confirm this assumption.

2.4.3 Genetic characterization of the bacteriocin

Primers P1 and P2 were used to amplify and sequence a 711-bp fragment (from 91 bp upstream of *pedA* to 33 bp downstream of the translational start of *pedC* [GenBank: M83924]) including the ribosome binding site as well as the -35/-10 region. The remaining sequence of the operon
Figure 2.1: Molecular weight determination of the bacteriocin produced by strain UVA1. (a) Coomassie stained SDS-PAGE gel. (b) SDS gel overlaid with the indicator strain *Listeria ivanovii* HPB28. Std: polypeptide molecular weight standard in kDa, F3c: active FPLC-fraction 10-fold concentrated. CFSc: cell-free supernatant of a UVA1 culture, 10-fold concentrated. CFS: cell-free supernatant not concentrated.
(2862 bp, from 13 bp upstream the end of *pedB* to the end of *pedD*) was amplified with primers pedopF and pedopR (Table A.1) and the generated DNA fragment was sequenced. This resulted in a 3473-bp sequence, comprising the four genes constituting the operon including the upstream region of *pedA* (91 bp), presumably containing the regulatory elements of the operon. The whole nucleotide sequence showed more than 99.5 % similarity to the published sequences for pediocin operons on the genomes of *P. acidilactici* K1 [GenBank: AY705375], *P. acidilactici* H [GenBank: U02482], *P. acidilactici* PAC1.0 [GenBank: M83924], *P. pentosaceus* [GenBank: AY316525], *Lactobacillus plantarum* [GenBank: AY316526], *P. parvulus* [GenBank: AY316524], and for coagulin on the genome of *Bacillus coagulans* [GenBank: AF300457]. Two sequences of 1009 and 1417 bp, upstream and downstream of the operon, respectively, were also PCR-amplified and sequenced. The resulting 5368-bp sequence was 99 % identical to the pediocin-encoding plasmid pSRQ11 described for *P. acidilactici* PA-1 (Motlagh *et al.*, 1994).

### 2.4.4 The antimicrobial activity is linked to the presence of the plasmid and the expression of *pedA*

Agarose gel electrophoresis of plasmid DNA preparation from strain UVA1 showed three bands: two extrachromosomal DNA elements at 9.5 kb and at >10 kb and one corresponding to chromosomal DNA, respectively (Figure 2.2 (a)). For strain UVA1-bac−, a non-inhibitory derivative of *P. acidilactici* UVA1 obtained after plasmid curing, in contrast, only chromosomal DNA was visible. Plasmid DNA prepared from the control pediocin producing strain UL5 exhibited the same bands as detected for UVA1, in addition to two larger extrachromosomal DNA elements.
Figure 2.2: Localization of the *pedA* gene on the plasmid by curing and Southern blotting. (a) Agarose gel electrophoresis of plasmid DNA. UVA1: *P. acidilactici* UVA1. UL5: *P. acidilactici* UL5. bac<sup>−</sup>: cured derivative of UVA1. M. Supercoiled DNA ladder in kb. ch: chromosomal DNA band. (b) Southern blot DNA hybridization of the DIG labeled *pedA*-probe with plasmidic DNA from *P. acidilactici* UVA1-bac<sup>−</sup>, UVA1 and UL5. (c) and (d) Agar-well diffusion assay with CFS of cultures of *P. acidilactici* UVA1 (c) and its cured derivative *P. acidilactici* UVA1-bac<sup>−</sup> (d).

Southern hybridization with the *pedA*-probe (Figure 2.2 (b)) yielded positive signals for the 9.5-kb and the >10-kb extrachromosomal elements present in UL5 and UVA1, but no hybridization occurred for UVA1-bac<sup>−</sup>,

2 *P. acidilactici* UVA1 and the pediocin gene *pedA*
confirming the presence of the pedA-gene on the 9.5-kb plasmid, the band at >10 kb probably being the relaxed circular form of the same plasmid.

Presence of the pedA-gene on the plasmid was confirmed by an agar-well diffusion assay, where the supernatant of a culture of UVA1-bac\(^-\) failed to exhibit inhibition of the indicator strain Listeria ivanovii HPB28 (Figure 2.2 (c) and (d)).

Furthermore, the pedA-transcript was detected by reverse-transcription-PCR on cDNA from P. acidilactici UVA1 (Figure 2.3, lanes 7-9) but not from the cured derivative UVA1-bac\(^-\) (Figure 2.3, lanes 1-3), providing the ultimate link between presence of the plasmid-localized genetic determinant and expression of the bacteriocin. The presence of a slight band at 100 bp for the non-plasmid containing strains (Figure 2.3, lanes 1-4) could not be explained, but the very weak intensity compared to lanes 7 to 9 does not represent a positive signal.

A second PCR, performed with primers P1 and P2 (Table A.1), which encompass the regulatory region upstream of the transcriptional start, resulted in no amplification in samples using cDNA as template, allowing us to exclude genomic DNA contamination in the RNA preparation of UVA1, UL5 and P. pentosaceus DSM20336\(^T\) (data not shown).

### 2.4.5 Distribution of pedA-containing strains in human faecal samples

The designed primers and probe allowed specific amplification of a 100-bp fragment located within the pedA-gene. As determined with spiked samples, the reaction was linear for concentrations ranging from 10\(^9\) to 10\(^5\) of P. acidilactici UVA1 cells per g of spiked faeces, corresponding to \(C_T\) values comprised between 22 and 35 (Figure 2.4 (a)).
Figure 2.3: Transcription analysis of pedA: pedA-reverse transcription-PCR on cDNA from \textit{P. acidilactici} UVA1-bac\textsuperscript{−} (1, 2, 3) or UVA1 (7, 8, 9) after 1 h 30, 2 h 30 and 3 h 30 of growth, respectively, and from \textit{P. acidilactici} DSM20284\textsuperscript{T} (4) or UL5 (6) and \textit{P. pentosaceus} DSM20336\textsuperscript{T} (5) after 2 h 30 of growth. 10: water instead of DNA. lm: low molecular weight DNA ladder (in bp). h: Tridyne 100-bp DNA ladder (in bp). Expected product size: 100 bp.

A \( C_T \) value of 35 was thus fixed as the upper limit for detection. Samples with a higher value were considered not to contain any pedA-gene. With this assay, the pediocin gene was detected in DNA isolated from 11 out of 13 children faecal samples tested, but in none of the 4 adult samples (Figure 2.4 (b)).
Figure 2.4: Detection of the pediocin gene pedA by real-time PCR on faecal DNA samples. (a) C<sub>T</sub> values of spiked samples plotted against spiked cell concentration in faecal DNA sample. (b) C<sub>T</sub> values obtained for children (C1-C13) and adult (A1-A4) faecal samples. F0: PCR amplification negative sample. w: water instead of DNA. Values represented are means and standard deviations for 3 repetitions.
2.5 Discussion

*P. acidilactici* UVA1 inhibited *L. ivanovii* HPB28 by production of a proteinaceous compound, as demonstrated by the total loss of activity after proteolytic treatments. This compound had a molecular weight of approximately 4.5 kDa, similar to pediocin AcH/PA-1. This was confirmed by the detection in strain UVA1 of a plasmid of approximately 9.5 kb, on which we could localize the *pedA* gene. We also found that the pediocin operon, which consists of the pediocin structural gene (*pedA*), the specific immunity gene (*pedB*), and genes required for processing, maturation and secretion of the bacteriocin (*pedC* and *D*), showed 99 to 100 % similarity to the published sequences for the pediocin AcH/PA-1 operon (Marugg *et al.*, 1992; Motlagh *et al.*, 1994). In all pediocin-producing strains isolated so far, the genetic determinants are plasmid-encoded (Rodriguez *et al.*, 2002). The comparison of genetic determinants of many pediocin-like producer strains (Miller *et al.*, 2005) showed that all pediocin genes were carried by plasmids, but the surrounding sequences on the plasmids can differ from one strain to the other. Sequencing data for the regions upstream the regulatory region and downstream the transcription terminator of the pediocin operon in UVA1 suggested that the plasmid harboured by *P. acidilactici* UVA1 is probably identical to pSRQ11, a 9.4 kb plasmid from *P. acidilactici* PA-1 (Marugg *et al.*, 1992).

Transcription analysis by reverse-transcription PCR using primers specific for the mRNA transcript of a particular protein is a straightforward method to establish the link between the presence of the genetic determinant and the observed protein activity. With this direct method, we could show that the *pedA*-transcript was synthesized in exponentially growing
cells of UVA1 but not in the cured, non-active derivative, UVA1-bac−, and that the inhibitory activity of UVA1 was due to pediocin production. It is also interesting to notice that in *P. pentosaceus* DSM20336T, the pedA-gene and the mRNA transcript were detected, but no inhibitory activity was observed against *L. ivanovii* HPB28 and the PCR with primers pedopF and pedopR did not yield any product (data not shown). Diep et al. (2006) reported similar observations in *P. pentosaceus* ATCC 25745, where a truncated pen locus lead to low expression of antimicrobial activity.

Pediococci are commonly associated with various plants and their products or meat (Carr et al., 2002; Simpson et al., 2002). There are only few reports on pediococci detected in human faeces or gastrointestinal tract (Millette et al., 2007; Barros et al., 2001; Walter et al., 2001), and only Millette et al. (2007), reported the presence of an antibacterial compound of proteinaceous nature, although not identified yet. Strain UVA1 is, to our knowledge, the first pediocin-producing *Pediococcus* to be isolated from human faecal material, which characteristic could be particularly interesting for the food industry for biopreservation as well as for possible probiotic effect. In this work, we showed, with a new real-time PCR assay, that strains containing the pedA-gene are relatively widespread in baby faecal material, but were not found in the 4 adult samples tested. Therefore, human baby faecal material could be a good source for isolating new pediocin-producing strains.
2.6 Conclusion

Data for the molecular size, sensitivity to protease and sequence of the plasmid-borne genetic determinant indicated that the antibacterial compound produced by *Pediococcus acidilactici* UVA1 is pediocin AcH/PA-1. To our knowledge, *P. acidilactici* UVA1 is the first pediocin-producing *Pediococcus* strain isolated from human faeces. Real-time PCR was an efficient method for detection of specific genes in faecal samples harbouring a complex microbiota. We showed a large distribution of *pedA*-containing strains in baby faecal samples.

After publication of these results, Millette and co-workers reported the identification of the bacteriocin produced by the intestinal strain *Pediococcus acidilactici* MM33 as the pediocin AcH/PA-1 (Millette *et al.*, 2008).
3 Detection and identification of *Bifidobacterium thermophilum* in human faeces by specific PCR and real-time PCR assays

Data presented in this chapter will be submitted for publication in BMC Microbiology in 2008
3 Detection of B. thermophilum in faeces by qPCR

3.1 Abstract

3.1.1 Background

Culture-independent methods based on the 16S ribosomal RNA sequence are nowadays widely used for assessment of the composition of the intestinal microbiota, in relation to host health or probiotic efficacy. Because B. thermophilum was only recently isolated from human faeces, no specific real-time PCR assay has been developed for detection and quantification of this species as component of the bifidobacterial community of the human intestinal flora.

3.1.2 Results

Design of specific primers and probe was achieved based on comparison of 108 published bifidobacterial 16S rDNA sequences with the recently published sequence of the human faecal isolate B. thermophilum RBL67. Specificity of the primer was tested in silico by similarity search against the sequence database and confirmed in vivo by PCR amplification on 17 Bifidobacterium strains, representing 12 different species, and two lactobacilli. The real-time PCR assay developed was linear for B. thermophilum RBL67 DNA quantities ranging from 0.02 ng/µl to 200 ng/µl and showed a detection limit of 10^5 cells per gram faeces. This test allowed to detect the presence of B. thermophilum in one among 17 human faecal samples tested. Additionally, selective plating experiments led to the purification of F9K9, a faecal isolate of human origin, which 16S rDNA sequence is 99.93 % similar to that of B. thermophilum RBL67.
3.1.3 Conclusion

Until recently, *B. thermophilum* was considered as a strain of animal origin, here we confirm its presence in the human gut and provide a new *B. thermophilum*-specific real-time PCR assay that can be used to further characterize the composition of the bifidobacterial community in the human gastrointestinal tract.

3.2 Background

Development of efficient tools is a prerequisite for a better knowledge of the complex gastrointestinal community composition, in particular in relation with diet, role in health and disease and modulation by probiotic or prebiotic consumption. As one of the predominant faecal component and because of their widespread use as probiotic organisms, bifidobacteria have attracted special interest in the study of the intestinal microflora. Traditionally, the intestinal bacterial community has been characterized from faecal samples by culture-based, microscopic, biochemical and physiological methods. However, culture-independent studies have established that only a fraction (20 to 40 %) of the organisms present in feces are cultivable (Suau *et al.*, 2001). The limits of traditional selective culture-based methods are particularly important for bifidobacteria because they have stringent nutrient requirement and usually grow poorly outside of the animal gut (Lamendella *et al.*, 2008).

Recent advances in molecular biology have led to the development of alternative, culture-independent methods (Zoetendal *et al.*, 2004). Two of the most widely used approaches for intestinal microbiota assessment are fluorescence *in situ* hybridization (FISH) and dot-blot hybridization
3 Detection of *B. thermophilum* in faeces by qPCR

(Gueimonde *et al.*, 2004; Blaut *et al.*, 2002). Both methods are based on the use of 16S ribosomal RNA (rRNA) encoding gene (16S rDNA) as target molecule. The rRNA sequences consist of alternating conserved and variable regions, variable regions contain information specific for different phylogenetic levels that can be exploited for the design of group-, genus-, species- or sub-species specific nucleic acid probes (Satokari *et al.*, 2003). Dot-blot hybridization, although simple and quick, often presents a high background noise and lacks sensitivity (Zoetendal *et al.*, 2004). FISH is probably the most advanced technique for enumerating faecal microbiota in terms of availability of specific probes (Vaughan *et al.*, 2005; Ben-Amor *et al.*, 2005), but it has the drawback of presenting low signal intensity and being time consuming and laborious (Gueimonde *et al.*, 2004).

For these reasons, new methods, using real-time PCR technology have been developed for rapid detection and/or enumeration of bacterial groups from large number of samples. Real-time PCR was demonstrated to have about 10 to 100 fold more sensitive detection than culture- and FISH-based enumeration techniques (Matsuki *et al.*, 2004), as well as to be rapid, easy and more accurate for quantification of low levels of bacteria (Gueimonde *et al.*, 2004; Carey *et al.*, 2007). This method has been used for the detection of many different species or groups of bacteria frequently isolated from human faeces (Matsuki *et al.*, 2004; Hopkins *et al.*, 2005; Rinttila *et al.*, 2004; de Preter *et al.*, 2008; Ott *et al.*, 2004). Of particular interest are the assays for specific detection or quantification of the genus *Bifidobacterium* (Penders *et al.*, 2005) as well as of bifidobacterial group or species (Gueimonde *et al.*, 2007; Malinen *et al.*, 2005). Many oligonucleotides were designed for bifidobacterial species found in the human intestinal tract, most of them based on the 16S rRNA sequence (Satokari
et al., 2003; Matsuki et al., 2003). Other target genes like the transaldolase encoding gene (Requena et al., 2002), heat-shock protein (HSP60) gene (Delcenserie et al., 2005) or the intergenic spacer of the 16S-23S rRNA gene (Haarman and Knol, 2005) are also being investigated for species-specific detection and quantification.

*Bifidobacterium thermophilum*, being considered as an animal-associated species, was never included in studies on the bifidobacterial composition of the human intestinal flora and, to our knowledge, no oligonucleotide was designed for the development of *B. thermophilum*-specific PCR or real-time PCR assay until now. Recently, design of a pair of oligonucleotides for PCR amplification of a portion of the 16S rDNA of *B. thermophilum* was reported, but effective specificity of the assay was questioned (Youn et al., 2008). In a previous work, strain RBL67 was identified as *B. thermophilum* using 16S rDNA sequence homology, comparative HSP60 sequence analysis, DNA-DNA genome hybridization and carbohydrate fermentation patterns (von Ah et al., 2007). This was the first demonstration of the presence of *B. thermophilum* in human faeces. In this study, we designed an oligonucleotide specific for *B. thermophilum* and used it for the elaboration of PCR and real-time PCR assays to study the distribution of this species in human faecal samples. In addition, we purified a new isolate from human infant faeces with 16S rDNA sequence showing high similarity to that of *B. thermophilum* RBL67.
3 Detection of *B. thermophilum* in faeces by qPCR

### 3.3 Material and methods

#### 3.3.1 Bacterial strains and culture conditions

Unless otherwise indicated, bifidobacteria and lactobacilli were grown in liquid cultures overnight in 10 ml MRSC medium consisting of MRS (de Man *et al.*, 1960), obtained from Biolife (Milan, Italy) and supplemented with 0.05 % L-cysteine hydrochloride, or on MRSC-agar plates (MRSC supplemented with 1.5 % w/v agar). Incubation was carried out for 24 h at 37 °C in anaerobic jars with an anaerobic atmosphere generation system (Oxoid AnaeroGen TM, Basel, Switzerland).

*B. thermophilum* RBL67, as well as *B. thermophilum* subsp. *suis* RBL68 and RBL70 are human infant faecal isolates (von Ah *et al.*, 2007; Touré *et al.*, 2003; Moroni *et al.*, 2006; Kheadr *et al.*, 2007). All other strains are commercial strains from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) or LMG (Laboratories for Microbiology and Microbial Genetics, Ghent, Belgium).

#### 3.3.2 Isolation of bifidobacteria from faecal samples

Twenty-one faecal samples from human adults (4) and children (17) were collected as already described (Mathys *et al.*, 2007). For efficient growth of *B. thermophilum* strains from faecal samples, Raffinose-Bifidobacterium (RB) (Hartemink *et al.*, 1996) and MRSC-NNLP (Dave and Shah, 1996) media were compared. Serial 10-fold dilutions of overnight cultures of *B. thermophilum* RBL67 (containing approximately $10^9$ cfu/ml) in saline solution (8.5 g/l NaCl, 1 g/l peptone, 0.05 % L-cysteine hydrochloride, pH 6-7) were plated on RB and on MRSC-NNLP, incubated 3 days anaerobically at 40 °C and cell counts were determined. Incubation tempera-
tture of 40 °C was chosen as an additional selective condition, due to the relative heat resistance of *B. thermophilum* spp. (von Ah *et al.*, 2007).

For isolation of bifidobacteria from faecal samples, 20 mg of samples were homogenized by vigorous vortexing in 200 µl of saline solution, 10-fold serially diluted in the same solution and spread on MRSC-NNLP agar plates. Plates were incubated for 3 days under anaerobic conditions at 40 °C and single isolates were observed under light microscope. Rod-shaped bacteria were selected for further analysis.

### 3.3.3 DNA purification methods

Total DNA was isolated from pure cultures of *B. thermophilum* RBL67, *B. thermophilum* DSM20210^T*, B. thermacidophilum* subsp. *porcinum* LMG21689^T*, *B. thermacidophilum* subsp. *thermacidophilum* LMG21395^T*, *B. breve* DSM20213^T* and *B. boum* DSM20432^T* according to Leenhouts *et al.* (1989).

Total DNA was prepared from 200 mg of 17 of the 21 faecal samples using the QiAamp DNA Stool Mini kit (Qiagen, Basel, Switzerland) according to manufacturer’s instructions. A PCR-amplification negative faecal sample (F0) was prepared by autoclaving one of the samples twice.

For determination of the detection limit, 10-µl aliquots of F0 were spiked before DNA preparation with a 10-fold serial dilution of *B. thermophilum* RBL67 (overnight culture in MRSC) at concentrations ranging from $10^9$ to 10 bacteria cells per g faeces. The extracted DNA was stored at -20 °C.
3 Detection of *B. thermophilum* in faeces by qPCR

### 3.3.4 DNA sequencing, PCR and qPCR reactions

Primers and probe used in this study were synthesized by Microsynth and are listed in Table A.1. Classical PCR was performed either on 2 μl DNA prepared from faecal samples as described above, or on 40 μl cell suspensions. For that, one colony was picked from an agar plate and resuspended in 210 μl of sterile, double distilled water. A 50-μl classical PCR reaction consisted of 2.5 U EuroTaq DNA Polymerase (Digitana, Horgen, Switzerland), 1.5 mM MgCl₂ (Digitana), 0.2 mM dNTP’s (GE Healthcare) and 0.5 μM of each primer. When DNA isolated from faecal samples was used as template, 0.1 μg/ml BSA was added to the PCR reaction. Amplification conditions were as follows: 3 min at 95 °C, 40 cycles of 15 sec at 95 °C, 30 sec at 62 °C and 2 min at 72 °C, followed by 7 min at 72 °C. Sequencing of the PCR product for 16S rDNA was performed by Microsynth (Balgach, Switzerland) using the primers btherm, 520F, 520R, 1100F, 1100R and lm3 (Table A.1).

Real-time PCR reactions were set in a total volume of 25 μl, containing 2.5 μl of DNA, 12.5 μl of qPCR MasterMix from Eurogentec (Seraing, Belgium), 0.3 μM of each primer and 0.1 μM of the TaqMan probe. Reactions were run on an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Rotkreuz, Switzerland). The amplification conditions were 2 min at 50 °C, 10 min denaturation at 95 °C, followed by 45 cycles of 15 sec at 95 °C and 1 min at 60 °C. The cycle threshold (Cₜ), corresponding to the number of cycles after which the target-DNA concentration increase becomes exponential, was monitored. Results were analyzed using the SDS 2.1 Software (Applied Biosystems). All reactions were done in triplicate and repeated at least twice (three times when faecal DNA extract was used as a template). Values in the text are means ± SD.
3.3 Material and methods

3.3.5 Design of a *B. thermophilum* specific primer and real-time PCR assay

One hundred and eight sequences of bifidobacterial 16S rDNA of more than 1 kb were retrieved from the EMBL nucleotide sequence database (http://www.ebi.ac.uk/embl) and were used, together with the sequence of the 16S rDNA of *B. thermophilum* RBL67 [GenBank: DQ340557], to prepare a multiple alignment with ClustalW in the sequence analysis program BioEdit (Hall, 1999). The primer btherm (Table A.1) was manually designed in a variable region.

Specificity of the primer was verified *in silico* with FASTA and the BLAST program from NCBI (version 2.2.15). For *B. thermophilum*-specific PCR amplification, primer btherm was used together with the bifidobacteria-specific reverse primer lm3. Cell suspensions of *B. thermophilum* RBL67 and DSM20210T, *B. thermacidophilum* strains RBL68, RBL70, LMG21395T and LMG21689T, *B. boum* DSM20432T, *B. breve* DSM20213T, *B. longum* DSM20219, *B. coryneforme* DSM2026T, *B. asteroides* DSMZ20089, *B. lactis* DSM10140, *B. animalis* DSM20105, *B. cuniculi* DSM20435T, *B. adolescentis* DSM20083T, *B. bifidum* DSM20456T, *Lb. delbrückii* subsp. *lactis* DSM200772T and *Lb. plantarum* DSM20174 were used as template for amplification with btherm and lm3 under the conditions described above to test the specificity of the PCR.

For the development of the real-time PCR assay, the btherm primer was modified to fit the lower melting temperature required for a TaqMan probe and adequate adjacent forward and reverse primers were designed with the program Primer3 (Rozen and Skaletsky, 2000) (Table A.1). Aliquots of 5 µl of DNA (20 ng/µl) isolated from pure cultures of *B. thermophilum* RBL67, *B. thermophilum* DSM20210T, *B. thermacidophilum*
subsp. *porcinum* LMG21689\(^T\), *B. thermacidophilum* subsp. *thermacidophilum* LMG21395\(^T\), *B. breve* DSM20213\(^T\) and *B. boum* DSM20432\(^T\) were amplified with this assay to assess its specificity. Localization of the primers btherm, bthermRTF and bthermRTR and of the TaqMan probe bthermTqM on an alignment of 16S rDNA sequences of seven bifidobacteria in shown in Figure 3.1.

**Figure 3.1:** Localization of the 16S rDNA targets for oligonucleotides designed in this study on a multiple alignment of 16S rDNA sequences of *B. thermophilum* RBL67 [GenBank: DQ340557.1], *B. thermophilum* ATCC25525 [GenBank: U10151.1], *B. thermacidophilum* subsp. *porcinum* LMG21689\(^T\) [GenBank: AY148470.1], *B. thermacidophilum* subsp. *thermacidophilum* LMG21395\(^T\) [GenBank: AB016246.1], *B. boum* JCM1211 [GenBank: D86190.1], *B. saeculare* DSM6533 [GenBank: D89330.1] and *B. breve* JCM1273 [GenBank: AF491832]. Numbers correspond to *E. coli* 16S rDNA positions.
3.4 Results

3.4.1 Design of a *B. thermophilum* specific PCR assay

Specificity of the *B. thermophilum* specific primer was assessed *in vivo* by PCR on colonies with primers btherm and the *Bifidobacterium*-specific primer lm3 (Table A.1). Of the 17 *Bifidobacterium* and two *Lactobacillus* strains tested, positive signals (amplification of a fragment of approximately 1.5 kb) were only obtained with the three faecal isolates *B. thermophilum* RBL67, *B. thermacidophilum* subsp. *suis* RBL68 and RBL70 (Figure 3.2, lanes 1 to 3, respectively), *B. thermophilum* DSM20210 (Figure 3.2, lane 6), and *B. thermacidophilum* subsp. *porcinum* LMG21689<sup>T</sup> (Figure 3.2, lane 5).

3.4.2 Detection of *B. thermophilum* in faecal DNA samples by PCR

Classical PCR analysis with the *B. thermophilum* specific primers btherm and lm3 on total DNA isolated from faecal samples spiked with known quantities of *B. thermophilum* RBL67 showed that the detection limit of the method was $10^8$ *B. thermophilum* cells per gram faeces (Figure 3.3).

This high detection limit did not allow DNA amplification from any of the 17 faecal samples. Efficacy of PCR amplification on faecal DNA samples was confirmed by amplification of a lm26/lm3 *Bifidobacterium*-specific 1.3 kb-fragment from each faecal DNA sample (data not shown).
Figure 3.3: Determination of the detection limit of the *B. thermophilum* PCR on faecal samples. PCR amplification with primers btherm and lm3 of DNA isolated from faecal sample F0, the PCR-amplification negative control (0) spiked with $10^9$ (9), $10^8$ (8), $10^7$ (7), $10^6$ (6) or $10^5$ (5) *B. thermophilum* RBL67 cells per gram faeces. h: Tridye 100-bp DNA ladder (in kb), t: Tridye 1-kb DNA ladder, in kb (New England Biolabs, Ipswich, MA, USA), w: water, 67: PCR on a colony of *B. thermophilum* RBL67.
3 Detection of *B. thermophilum* in faeces by qPCR

### 3.4.3 Development of a real-time PCR assay for detection of *B. thermophilum* in human faeces

Real-time PCR was chosen as an alterative to the classical PCR for its higher sensitivity. *B. thermophilum* specificity of the real-time PCR performed with primers bthermRTF, bthermRTR and the TaqMan probe bthermTqM was tested by amplification of DNA isolated from six different *Bifidobacterium* strains from four closely related species.

A positive signal was obtained for *B. thermophilum* RBL67 (C_T = 17.3 ± 0.5), *B. thermophilum* DSM20210^T^ (C_T = 24.9 ± 0.3) and the closely related species *B. thermacidophilum* subsp. *porcinum* LMG21689^T^ (C_T = 16.3 ± 0.4), but not for *B. thermacidophilum* subsp. *thermacidophilum* LMG21395^T^, *B. breve* DSM20213^T^, and *B. boum* DSM20432^T^.

Amplification of DNA from *B. thermophilum* RBL67 with this new assay was shown to be linear for DNA concentrations ranging from 0.02 ng/µl (C_T = 28.1 ± 0.3) to 200 ng/µl (C_T = 15.3 ± 0.4) with a regression coefficient R^2^ = 0.991 (data not shown).

### 3.4.4 Screening of faecal samples with qPCR

As for the classical PCR approach, the detection limit of the real-time PCR assay was determined by analysis of DNA isolated from spiked faecal samples and was equal to 10^5^ bacterial cells per gram of faeces.

Detection in faecal samples was shown to be linear between 10^9^ and 10^5^ cells per gram of faeces and corresponded to C_T values comprised between 21.6 ± 0.6 and 33.8 ± 0.1, respectively, with a regression coefficient R^2^ = 0.995 (data not shown).
One of the 17 children faecal samples (sample C7) gave a positive signal within this range (Figure 3.4) with a $C_T$ value of 28.6 ± 0.3, corresponding to a concentration of $5 \times 10^6$ cells per gram faeces.

**Figure 3.4:** Detection of *B. thermophilum* in faecal samples by real-time PCR targeting 16S rDNA. Cycle threshold ($C_T$) values measured for faecal samples from 13 children (C1 to C13) and 4 adults (A1 to A4). F0: PCR-negative faecal sample, w: water instead of DNA, RBL67: DNA from pure culture of RBL67, 21395: DNA from *B. thermacidophilum* subsp. *thermacidophilum* LMG21395$^T$. Values are means and standard deviations for three repetitions of the real-time PCR assay with three replicates each.
3 Detection of *B. thermophilum* in faeces by qPCR

### 3.4.5 Isolation of bifidobacteria from faecal samples

Raffinose-Bifidobacterium (RB) medium and MRSC-NNLP were tested for isolation of new *B. thermophilum* strains from human faeces. MRSC-NNLP was chosen for isolation of bifidobacteria because it allowed a better growth of RBL67 (1.5x10^9 cfu/ml after three days incubation at 40 °C, in comparison to 2.9x10^7 cfu/ml in RB-medium and under the same conditions) and was easier to prepare. Approximately 10^2 colonies per microgram faeces could be cultivated from all the 21 samples plated on MRSC-NNLP agar. Microscopic observations of the isolates showed that the medium was not completely selective, allowing growth of non rod shaped microorganisms.

### 3.4.6 PCR identification of a *B. thermophilum* faecal isolate

After microscopic observation of the MRSC-NNLP-isolates, 60 rod-shaped microorganisms were selected for PCR analysis on colony. Twenty-five of the 60 colonies tested were positive with the *Bifidobacterium* genus specific primers lm26/lm3, but only one of them gave a positive signal with btherm/lm3. This isolate, F9K9, was streaked several times on MRSC agar and the absence of contaminant other than *Bifidobacterium* was confirmed by three PCR reactions with lm26/lm3, btherm/lm3 and bak4/bak11w (Schürch, 2002) (data not shown). Sequencing of the 16S rDNA fragment amplified with lm26 and lm3 yielded a 1454-bp sequence which was 99.93 % identical to the 16S rDNA of *B. thermophilum* RBL67. Sequence identities with other *Bifidobacterium* strains are summarized in Table 3.1.
Table 3.1: 16S rDNA sequence identities of isolate F9K9 with published 16S rDNA sequences. The percentage of identity (% I) was determined by comparison of the sequence of F9K9 against the sequences present in the database with the BLAST tool from NCBI.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>% I</th>
</tr>
</thead>
<tbody>
<tr>
<td>DQ340557.1</td>
<td>B. thermophilum RBL67</td>
<td>99.93%</td>
</tr>
<tr>
<td>AY148470.1</td>
<td>B. thermacidophilum subsp. porcinum LMG 21689T</td>
<td>99.64%</td>
</tr>
<tr>
<td>D86190.1</td>
<td>B. boum JCM 1211</td>
<td>98.15%</td>
</tr>
<tr>
<td>AB016246.1</td>
<td>B. thermacidophilum subsp. thermacidophilum LMG 21395T</td>
<td>97.02%</td>
</tr>
<tr>
<td>D89330.1</td>
<td>B. saeculare DSM 6533</td>
<td>95.82%</td>
</tr>
<tr>
<td>AF491832</td>
<td>B. breve JCM 1273</td>
<td>95.29%</td>
</tr>
<tr>
<td>U10151.1</td>
<td>B. thermophilum ATCC 25525</td>
<td>95.16%</td>
</tr>
</tbody>
</table>

3.5 Discussion

Real-time PCR is known to be a more sensitive technique than classical PCR. This is reflected by our results for specific amplification of 16S rDNA from spiked faecal samples, where changing from classical PCR to real-time PCR for the detection of B. thermophilum in faecal samples lowered the detection limit of the assay from $10^8$ to $10^5$ cells per gram faeces. The high sensitivity obtained for real-time PCR in this study is similar to detection limits reported by different groups for other Bifidobacterium species or genus specific real-time PCR assays. Matsuki et al. (2004), Penders et al. (2005) and Gueimonde et al. (2004), for example, reported detection limits of $10^6$, $5\times10^3$ and $5\times10^4$ cells of Bifidobacterium spp. per gram faeces, respectively.

B. thermacidophilum was first described as a new species by Dong et al. (2000) who reported its isolation from waste water. Based on
Detection of *B. thermophilum* in faeces by qPCR

16S rDNA analysis, they found that *B. thermacidophilum* was relatively close to *B. thermophilum* (92.7 % similarity) and that these two species formed a distinct phylum relative to other *Bifidobacterium* species. Zhu *et al.* (2003) detected *Bifidobacterium* isolates from piglet faeces for which they could not assign a species. The isolates showed high homology to *B. thermacidophilum*, *B. thermophilum* and *B. boum*, according to 16S rDNA and HSP60 analysis, but discrepancies in phenotypic characteristics and DNA-DNA hybridization. Finally the authors suggested to classify them as *B. thermacidophilum* subsp. *porcinum* as a new subspecies. This proposition was later questioned by other authors (von Ah *et al.*, 2007). Later on, Moroni *et al.* (2006) and Kheadr *et al.* (2007) assigned the faecal isolates *Bifidobacterium* sp. RBL68 and RBL70 to this new subspecies (*B. thermacidophilum* subsp. *suis* is a synonym for *B. thermacidophilum* subsp. *porcinum*), based on 16S rDNA similarities with the sequence published by Zhu *et al.* (2003).

Amplification of 16S rDNA fragments with both PCR and real-time PCR assays developed in this study showed high specificity, occurring only with DNA from *B. thermophilum* and *B. thermacidophilum* strains, but not from *B. boum*, a species which is often phylogenetically associated with *B. thermacidophilum* and *B. thermophilum* (Felis and Dellaglio, 2007).

Interestingly, with PCR as well as with real-time PCR, amplification occurred for the subspecies *porcinum/suis* (LMG21689<sup>T</sup>, RBL68 and RBL70) of *B. thermacidophilum*, but not for the subspecies *thermacidophilum* (LMG21395<sup>T</sup>). These results are in accordance with the observation made by von Ah *et al.* (2007) who compared the 16S rDNA sequence of RBL67 to other bifidobacteria and found the highest per-
percentage of identity (99 %) with *B. thermacidophilum* subsp. *porcinum* LMG21689\textsuperscript{T} isolated by Zhu *et al.* (2003). This percentage was 96 % for *B. thermacidophilum* subsp. *thermacidophilum* LMG21395\textsuperscript{T} and 94 % for the *B. thermophilum* type strain. Taken together, these data question whether strain LMG21689\textsuperscript{T} has been correctly classified and express again the need for a complete phylogenetic analysis for accurate classification of the *boum/thermophilum/thermacidophilum* branch with sequence data from more strains of these closely related species.

Until now, *B. thermophilum* was considered as an animal-associated species, mainly present in faeces of ruminants and pigs. The amplification of a specific 16S rDNA sequence with our real-time PCR on the children faecal sample C7 as well as the isolation of a *B. thermophilum* isolate from human faeces during this work support the assumption of von Ah *et al.* (2007) that the presence of *B. thermophilum* in food cannot be used to discriminate between animal and human bacterial contamination, as previously suggested by Delcenserie *et al.* (2004).

### 3.6 Conclusion

This is the first report of the development of a real-time PCR assay for specific detection of *B. thermophilum*, a species that was not included in analysis of the composition of the bifidobacterial human intestinal microflora until now. Using this assay, we detected *B. thermophilum* at a concentration of $5 \times 10^6$ cells per gram in one faeces sample, confirming the presence of this species in human faecal material.
4 Antibiotic resistance profile of *Bifidobacterium thermophilum* RBL67 and molecular genetic approaches for identification of its bacteriocin gene
4.1 Abstract

The occurrence of transferable antibiotic resistance determinants is a serious safety issue in strains for food and probiotic applications. Furthermore, data obtained with phenotypic antibiotic resistance screening are difficult to compare between different studies due to lack of standardized techniques and breakpoint definitions. To circumvent these limitations, the possible presence of antibiotic resistance genes in *Bifidobacterium thermophilum* RBL67 was assessed using microarray hybridization. Using this approach, the phenotypic resistance of *B. thermophilum* RBL67 against tetracycline was shown to be due to the *tet(W)* gene.

In a previous work, thermophilicin B67, a bacteriocin produced by *B. thermophilum* RBL67 could not be purified using standard bacteriocin purification procedures. Therefore, a molecular genetic method for the identification of the bacteriocin-encoding gene was attempted. In this work, we used an approach based on plasmid pFUN, originally developed for *Lactococcus lactis*. As a first step towards identification of the export signal sequences in the genome of this strain, *B. thermophilum* RBL67 genomic libraries of translational fusions to the Nuc-encoding gene devoid of its export signal were constructed in pFUN and established in *E. coli*.

4.2 Background

4.2.1 Determination of antibiotic resistances in lactic acid bacteria and bifidobacteria

Due to important use of lactic acid bacteria (LAB) as starter cultures in fermentative processes and bifidobacteria as probiotic organisms, large
amounts of these bacteria are being consumed through diet. Therefore, safety aspects of theses microorganisms, including the presence of potentially transferable antibiotic resistances, and the associated risk of transfer of these resistances to human commensal or pathogenic bacteria have to be carefully assessed (FAO/WHO, 2002).

The tetracycline resistance gene \textit{tet}(W), encoding ribosomal protection protein, is of particular concern for safety of probiotic strains, mainly because transferability of this gene is strongly suspected (Kazimierczak \textit{et al.}, 2006). Indeed, \textit{tet}(W) was originally identified in the gut commensal \textit{Butyrivibrio fibrisolvens}, where it was associated with the conjugative transposon Tn\textit{B1230} (Melville \textit{et al.}, 2004), and this gene exhibited high frequency transfer (Scott \textit{et al.}, 1997).

In addition, \textit{tet}(W) was shown to be widespread among anaerobic commensal gut bacteria (Scott \textit{et al.}, 2000), including \textit{Fusobacterium prausnitzii}, one of the dominant human colonic anaerobe (Suau \textit{et al.}, 2001), and gene sequences from different isolates were shown to be remarkably conserved, suggesting recent gene transfer events (Scott \textit{et al.}, 2000).

In bifidobacteria, \textit{tet}(W) was recently detected by microarray analysis in \textit{B. lactis} strains isolated from probiotic milk products (Kastner \textit{et al.}, 2006), and by PCR in seven \textit{B. animalis} subsp. \textit{lactis} and \textit{B. bifidum} probiotic isolates (Masco \textit{et al.}, 2006). The \textit{tet}(W) gene was also identified in 16 tetracycline resistant \textit{Bifidobacterium} isolates from human fecal samples (Flórez \textit{et al.}, 2006).

In this work, the antibiotic resistance profile of the potentially probiotic bacteriocin producing strain \textit{B. thermophilum} RBL67 was assessed by phenotypic susceptibility testing against chosen common antibiotics as well as by microarray hybridization combined with PCR.
4.2 Background

4.2.2 Approaches for identification of the bacteriocin encoding gene in *B. thermophilum* RBL67

Classical procedure for identification of a bacteriocin relies on purification of the protein as a first step. Several attempts were made to adapt well described bacteriocin purification methods to thermophilin B67, a bacteriocin produced by *B. thermophilum* RBL67, but were not successful due to a loss of activity during the last purification steps (von Ah, 2006). Alternative approaches rely on the direct identification of bacteriocin-encoding genes by molecular genetic methods. In this work, three possibilities were considered.

The first idea was to identify the gene responsible for bacteriocin production by knock-out random transposon mutagenesis. Unfortunately, due to a lack of efficient techniques for introduction of foreign DNA into bifidobacteria and methods for selection and identification of mutants knocked-out in the bacteriocin-encoding gene, this technique was not applicable in our case.

The second approach was based on the observation that bacteriocins, as for example pediocin, are often plasmid encoded (Rodriguez et al., 2002). If this was also the case for the bacteriocin produced by *B. thermophilum* RBL67, plasmid isolation and sequencing, followed by sequence similarity searches would be a straightforward procedure to identify the bacteriocin-encoding gene.

The third approach presented here exploits the fact that bacteriocin genes encode proteins meant to be exported outside of the producer cell. The method used relied on heterologous construction of a gene bank of the target organism and translational fusion to a reporter gene devoid of signal sequence region for detection of exported proteins.
As described in the general introduction of this thesis, this kind of approach has a long history of use for identification of genes involved in protein secretion and the analysis of signal sequence structure and function (Payne and Jackson, 1991). It presents the advantage that DNA manipulations and activity screening are done in DNA manipulation-friendly microorganisms such as *E. coli* and *L. lactis*, circumventing the difficulties of application of molecular genetic methods in bifidobacteria.

For this approach, we used the method developed by Poquet et al. (1998), in which exported proteins are detected after construction of a genomic library of the organism of interest in the plasmid pFUN. It contains upstream of the reporter staphylococcal nuclease gene, a multiple cloning site in which DNA fragments can be cloned in-frame for identification of export signal sequences (Poquet et al., 1998). It has been used successfully by MacConaill et al. (2003) to investigate protein export in *B. breve* UCC2003. Similar approaches were also used for protein secretion studies in mycobacteria (Downing et al., 1999) and in *L. lactis* (Ravn et al., 2000).

In this chapter, different characteristics of *B. thermophilum* RBL67, such as identification of antibiotic resistances and detection of extrachromosomal elements are presented. In addition, the construction of genomic libraries of *B. thermophilum* RBL67 in pFUN is reported, as a first step towards identification of exported proteins, among which the bacteriocin thermophilicin B67.
4.3 Material and methods

4.3.1 Plasmids, bacterial strains and growth conditions

Unless otherwise indicated, bifidobacteria were grown in liquid cultures overnight in 10 ml MRSC medium consisting of MRS (de Man et al., 1960), obtained from Biolife (Milan, Italy) and supplemented with 0.05 % L-cysteine hydrochloride or on MRSC-agar plates (MRSC supplemented with 1.5 % w/v agar) for 24 h at 37 °C and in anaerobic jars with an atmosphere generation system (Oxoid AnaeroGen TM, Basel, Switzerland).

*Lactococcus lactis* AC1 was used as a positive control for plasmid isolation and propagated in M17 (Biolife, Milan, Italy). *B. asteroides* DSM20089T was also used as positive control for plasmid isolation and was a commercial strain from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). *B. thermophilum* RBL67, as well as *B. thermacidophilum* subsp. *suis* RBL68 and RBL70 are human infant faecal isolates (Touré et al., 2003; von Ah et al., 2007; Moroni et al., 2006; Kheadr et al., 2007). *B. pseudolongum* DSM20092 and *B. thermophilum* DSM20210 were obtained from DSMZ and were used in the antibiotic resistance profile determination as positive and negative controls for presence of the *tet(W)* gene, respectively, according to results published by Mayrhofer et al. (2007). *B. longum* DSM20219, *B. lactis* DSM10140, *B. animalis* DSM20104 and *Lb. reuteri* SD2112 (ATCC55730) are type strains and were additionally used for the antimicrobial discs susceptibility testing.

*E. coli* DH10B (genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZ ΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ-
rpsL nupG) competent cells were used as host for construction of the gene bank and were grown in Luria-Bertani medium (LB, Becton Dickinson, Franklin Lakes, NJ, USA) at 37 °C under agitation. When needed, LB medium was supplemented with 100 µg/ml ampicilin sodium salt (Applichem, Darmstadt, Germany) for maintaining of the plasmid.

4.3.2 DNA manipulations

Isolation of extrachromosomal elements from *B. thermophilum* RBL67, *B. thermacidophilum* subsp. *suis* RBL68 and RBL70, *B. asteroides* DSM 20089T and *Lc. lactis* AC1 was performed as described in section 2.3.6. The DNA was visualized after electrophoresis on a 0.8 % agarose gel in 1x TAE buffer at 100 V for 1.5 h. The supercoiled DNA ladder (Promega, Madison WI, USA) was used as size standard.

For detection of the *tet*(W)-gene, PCR was performed with primers tetW-FW and tetW-RV (Table A.1). Amplification conditions were 5 min at 95 °C followed by 35 cycles of: 1 min at 95 °C, 1 min at 64 °C and 1 min at 72 °C with a final polymerization step 8 min at 72 °C.

For control of completeness of the genomic libraries, PCR analyses were performed on plasmid preparations with primers ForTal and RevTal, lm26 and lm3 and PyrFor and PyrRev (Table A.1) for detection of genes encoding transaldolase, 16S rRNA and pyruvate kinase, respectively, under amplification conditions described by Requena *et al.* (2002), Satokari *et al.* (2003) and Vaugien *et al.* (2002), respectively. The PCR reactions contained either 40 µl cell suspension or 1 µl plasmid preparation, 1.5 mM MgCl₂, 2.5 U Taq polymerase (Digitana, Horgen, Switzerland), 0.2 mM dNTP’s (GE Healthcare, Buckinghamshire, England) and 0.5 µM of each primer.
4.3 Material and methods

PCR products were visualized after electrophoresis on a 0.8 % agarose gel in 1 x TAE buffer at 100 V for 1 h. The low molecular weight DNA ladder (New England Biolabs, Ipswich, MA, USA) was used as size standard.

4.3.3 Phenotypic antibiotic resistance profile

*B. longum* DSM20219, *B. lactis* DSM10140, *B. pseudolongum* DSM20092, *B. animalis* DSM20104, *B. thermophilum* DSM20210 and RBL67, *B. thermacidophilum* subsp. *suis* RBL68 and RBL70 and *Lb. reuteri* SD2112 were grown overnight in 10 ml MRSC broth, the cultures were diluted in reduced peptone water to an optical density at 600 nm of 0.25 and spread with a cotton swab on lactic acid bacteria susceptibility test medium supplemented with L-cysteine hydrochloride (LSM-C, Klare et al. (2005)).

Commercially available antibiotic discs (BioMérieux, Marcy l'Etoile, France) were applied on the surface of the agar and plates were incubated 24 h at 37 °C anaerobically. Diameter of the inhibition zone was measured in mm, including the diameter of the disc (8 mm). The experiment was repeated twice with *B. thermophilum* RBL67 and diameters of inhibition didn’t differ of more than 10 % between the repetitions.

4.3.4 Microarray hybridization for detection of antibiotic resistance genes

*B. thermophilum* RBL67, *B. pseudolongum* DSM20092 and *B. thermophilum* DSM20210 were screened for 90 different known antibiotic resistance genes on the microarray spotted with specific oligonucleotides (Clondiag, Jena, Germany), following the protocol described by Perreten et al. (2005). This involved randomly primed amplification of DNA fol-
followed by 2 subsequent polymerization reactions and enzymatic biotin-labeling, hybridization of labeled DNA onto the array. Data were analyzed using IconoClust software (Clondiag).

**4.3.5 Construction of genomic libraries of *B. thermophilum* RBL67**

Restriction endonucleases, T4 DNA ligase, and antarctic phosphatase were purchased from New England Biolabs (Ipswich, MA, USA) and were used as recommended by the manufacturer. Large-scale production of total DNA from 1 l culture of *B. thermophilum* RBL67 was performed as described by Leenhouts et al. (1989), with modifications suggested by Meile et al. (2001). Total DNA was partially digested by incubation with Sau3A and the enzyme was heat inactivated (65 °C for 5 min). Three *B. thermophilum* RBL67 genomic libraries were constructed by cloning *B. thermophilum* DNA fragments ranging in size from 0.5 to 3.0 kb into each of the unique dephosphorylated BamHI, BclI, or BglII sites of pFUN, respectively. Ligations were performed according to standard procedures (Sambrook and Russel, 2001). An aliquot of 1 µl of each ligation mixture was introduced into *E. coli* DH10B by electroporation. Preparation of *E. coli* DH10B competent cells was performed following standard procedures (Sambrook and Russel, 2001) and electroporation was performed with an Equibio apparatus (Witec AG, Littau, Switzerland) under following conditions: 25 µF, 200 Ω, and 2.5 kV/cm. SOC medium (Sambrook and Russel, 2001) was added to the cells and the mixture was incubated at 37 °C for 1 h. Selection of the transformants was achieved by plating on LB medium supplemented with ampicilin (100 µg/ml) and incubating overnight at 37 °C.
4.3 Material and methods

To confirm the size of the inserted DNA fragments, nine transformants were randomly picked and plasmids were isolated using the CTAB method. Briefly, transformants were grown overnight in 3 ml LB with ampicilin (100 µg/ml). Cells from 1.5 ml of the cultures were harvested, lysed for 5 min in 200 µl STET buffer (8 g/l sucrose, 0.1 ml/l Triton-X, 50 mM EDTA, 50 mM Tris-HCl, pH 8) with 0.2 mg lysozyme and boiled for 40 s. Cell debris were removed with a toothpick after centrifugation for 15 min at 13’000 x g. Plasmid DNA was precipitated by adding 8 µl CTAB 5 % (cetyltrimethyl ammonium bromide) and centrifuging 5 min at 13’000 x g. 300 µl 1.2 M NaCl and 750 µl 100 % ethanol were added, samples were centrifuged for 15 min at 13’000 x g, washed once with 70 % ethanol, vacuum dried and resuspended in 30 µl water. For excision of the insert, plasmids were restricted with enzymes XbaI and SmaI under conditions recommended by the supplier. Restriction digests were visualized after electrophoresis on a 0.8 % agarose gel in 1 x TAE buffer at 100 V for 1 h. The Tridye 1-kb and Tridye 100bp DNA ladder (New England Biolabs, Ipswich, MA, USA) were used as size standards. Finally, plasmid DNA was prepared from approximately 10’000 *E. coli* clones for each genomic library by using either the FlexiPrep Kit (GE Heathcare) or the Plasmid Midi Kit from Qiagen (Basel, Switzerland).
4 Antibiotic resistance of RBL67 and search of its bacteriocin gene

4.4 Results

4.4.1 Phenotypic determination of the antibiotic resistance profile of *B. thermophilum* RBL67

In order to determine antibiotic resistance profile of *B. thermophilum* RBL67, sensibility of this strain as well as seven other bifidobacteria and *Lb. reuteri* to 15 antibiotics was assessed by antibiotic discs (Table 4.1). Most of the bifidobacteria tested were not or only slightly inhibited by gentamycin, neomycin, kanamycin, streptomycin, nalidixic acid and mupirocin. Additionally, *B. thermophilum* RBL67 and DSM20210 as well as *B. lactis* DSM10140, the faecal isolates *B. thermacidophilum* subsp. *suis* RBL68 and RBL70 and *Lb. reuteri* S2112 showed small or inexistent diameter of inhibition against tetracycline and oxytetracycline. On the other hand, all strains tested exhibited large diameters of inhibition against erytromycin. *B. thermophilum* RBL67, as well as *B. lactis* DSM10140, *B. animalis* DSM20104 and *B. longum* DSM20219 were inhibited by the antibiotics vancomycin, rifampicin, chloramphenicol, penicilin G and ampicilin (Table 4.1).

4.4.2 Strain RBL67 carries the tet(W) gene

For genotypic confirmation of these observations, total DNA of *B. thermophilum* RBL67, *B. pseudolongum* DSM20092 and *B. thermophilum* DSM20210 were subjected to genetic screening for 90 known resistance genes by microarray hybridization. Clear positive signal was produced by the tetracycline resistance gene tet(W) in both *B. thermophilum* strains but not in *B. pseudolongum* (Figure 4.1).
Table 4.1: Antibiotic resistance profiles of *Bifidobacterium* strains and *Lb. reuteri* obtained by antibiotic susceptibility discs. ni: no inhibition halo; -: not determined; numbers are inhibition diameters in mm, disc included (diameter of the disc: 8 mm)

<table>
<thead>
<tr>
<th>Antibiotic (quantity/disc)</th>
<th><em>B. thermophilum</em> RBL67</th>
<th><em>B. thermacidophilum</em> RBL68</th>
<th><em>B. thermacidophilum</em> RBL70</th>
<th><em>B. thermophilum</em> DSM20102</th>
<th><em>B. lactis</em> DSM10140</th>
<th><em>B. animalis</em> DSM20104</th>
<th><em>B. longum</em> DSM201029</th>
<th><em>B. pseudolongum</em> DSM20092</th>
<th><em>Lb. reuteri</em> SD2112</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline (30 μg)</td>
<td>14</td>
<td>ni</td>
<td>15</td>
<td>ni</td>
<td>19</td>
<td>29</td>
<td>33</td>
<td>35</td>
<td>ni</td>
</tr>
<tr>
<td>Oxytetracycline (30 μg)</td>
<td>13</td>
<td>9</td>
<td>16</td>
<td>ni</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>35</td>
<td>ni</td>
</tr>
<tr>
<td>Gentamycin (120 μg)</td>
<td>ni</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ni</td>
<td>ni</td>
<td>11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Neomycin (10 μg)</td>
<td>ni</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kanamycin (30 μg)</td>
<td>ni</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ni</td>
<td>ni</td>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Streptomycin (10 μg)</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ni</td>
<td>ni</td>
<td>11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nalidixic acid (30 μg)</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
</tr>
<tr>
<td>Mupirocin (200 μg)</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>-</td>
<td>-</td>
<td>ni</td>
<td>ni</td>
</tr>
<tr>
<td>Erytromycin (15 μg)</td>
<td>36</td>
<td>35</td>
<td>34</td>
<td>40</td>
<td>38</td>
<td>37</td>
<td>18</td>
<td>45</td>
<td>23</td>
</tr>
<tr>
<td>Novobiocin (30 μg)</td>
<td>17</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>16</td>
<td>20</td>
<td>13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vancomycin (30 μg)</td>
<td>28</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25</td>
<td>25</td>
<td>23</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rifampicin (5 μg)</td>
<td>26</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>22</td>
<td>23</td>
<td>25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chloramphenicol (30 μg)</td>
<td>36</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>37</td>
<td>36</td>
<td>35</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Penicilin G (10 IU)</td>
<td>45</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>32</td>
<td>38</td>
<td>35</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ampicilin (10 μg)</td>
<td>43</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>32</td>
<td>40</td>
<td>30</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 4.1: Antibiotic resistance profile of Bifidobacterium strains. Microarrays hybridized with genomic DNA of B. thermophilum RBL67 (a), B. pseudolongum DSM20092 (b) and B. thermophilum DSM20210 (c). Spots marked with a frame: tet(W). C: position control reaction, S: B. lactis 16S rDNA. Unmarked single spots result from unspecific hybridization.

PCR on colonies with primers specific for the tet(W) resistance gene and gel electrophoresis showed amplicons of the expected size (168 bp) for the three human isolates B. thermophilum RBL67, B. thermacidophilum
subsp. suis RBL 68 and RBL70 (Figure 4.2, lanes 1, 2 and 3, respectively) and for the tet(W)-gene containing strains *Lb. reuteri* SD2112, *B. thermophilum* DSM20210 and *B. lactis* DSM 10140 (Figure 4.2 lanes 4, 5 and 6, respectively) but not for the non tet(W)-containing strain *B. pseudolongum* DSM20092 (Figure 4.2, lane 7).

**Figure 4.2:** Detection of *tet*(W) by PCR amplification. Gel electrophoresis of PCR amplification with *tet*(W) specific primers on colonies from *B. thermophilum* RBL67 (1), *B. thermacidophilum* subsp. *suis* RBL70 (2), *B. thermacidophilum* subsp. *suis* RBL68 (3), *Lb. reuteri* SD2112 (4), *B. thermophilum* DSM20210 (5), *B. lactis* DSM10140 (6) and *B. pseudolongum* DSM20092 (7). w: water. l: low molecular weight DNA marker, in bp (New England Biolabs, Ipswich, MA, USA).
4 Antibiotic resistance of RBL67 and search of its bacteriocin gene

4.4.3 *B. thermophilum* RBL67 harbors no plasmid

Plasmids were isolated from the three *Bifidobacterium* faecal isolates RBL67, RBL68 and RBL70 as well as from the plasmid containing strains *Lc. lactis* AC1 and *B. asteroides* DSM20089<sup>T</sup>. Gel electrophoresis showed presence of eight bands of size ranging from 2.4 to more than 10 kb for *Lc. lactis* AC1, whereas one band corresponding to the 2.1-kb plasmid pAP1 was visible for *B. asteroides* DSM20089<sup>T</sup> (Figure 4.3). But for the three *Bifidobacterium* faecal isolates RBL67, RBL68 and RBL70, no extra-chromosomal element could be detected.

**Figure 4.3:** Agarose gel electrophoresis of plasmid DNA prepared from *Lc. lactis* AC1, *B. asteroides* DSM20089<sup>T</sup> and the *Bifidobacterium* faecal isolates RBL68, RBL70 and RBL67. M: Supercoiled DNA ladder in kb.
4.4.4 Construction of genomic libraries of *B. thermophilum* RBL67 in pFUN and establishment in *E. coli* DH10B

Three genomic libraries of *B. thermophilum* RBL67 were constructed in the three cloning sites of pFUN, allowing translational fusions between inserted *B. thermophilum* DNA fragments of sizes ranging from 0.5 to 3 kb and the reporter gene $\Delta_{SP} \text{Nuc}$ in the three reading frames (Figure 4.4).

![Agarose gel electrophoresis of plasmids isolated from nine randomly picked transformants (1-9) and restricted with enzymes XbaI and SmaI. nr: no restriction control. 1kb, 100: Tridy 1-kb and 100bp DNA markers, respectively, in kb (New England Biolabs, Ipswich, MA, USA). Bands at 8 kb represent the plasmid pFUN, inserted DNA fragments vary in sizes from 0.5 to 3 kb.](image)

**Figure 4.4:** Agarose gel electrophoresis of plasmids isolated from nine randomly picked transformants (1-9) and restricted with enzymes XbaI and SmaI. nr: no restriction contol. 1kb, 100: Tridy 1-kb and 100bp DNA markers, respectively, in kb (New England Biolabs, Ipswich, MA, USA). Bands at 8 kb represent the plasmid pFUN, inserted DNA fragments vary in sizes from 0.5 to 3 kb.
Plasmids were prepared from approximately 10’000 *E. coli* clones from each of the libraries and PCR amplification with primers targeting three conserved bifidobacterial genes were achieved on each plasmid preparation to assess completeness of the genomic libraries. A fragment of the transaldolase encoding gene (primers ForTal and RevTal, Table A.1) and the 16S rDNA (primers lm3 and lm26, Table A.1) were detected in plasmid prepared from each of the libraries, whereas the gene for pyruvate kinase (primers PyrFor and PyrRev, Table A.1) was found only for the genomic library constructed in the *Bam*H1 cloning site of pFUN (data not shown). All in all, each of the tested genes was detected at least in one of the three genomic libraries.

**4.5 Discussion**

Phenotypic susceptibility testing is widely used for rapid determination of antibiotic resistance profiles. However, these data are dependant on bacterial growth, media and method used. Moreover, standard procedures and breakpoints are poorly validated for susceptibility testing of LAB and bifidobacteria, making it difficult to compare data obtained by different groups. But generally, it has been observed that most bifidobacteria species are intrinsically resistant to the amylogylcosides gentamycin, neomycin, kanamycin and streptomycin, to the Gram-negative spectrum antibiotics fusidic acid and nalidixic acid, and to mupirocin. In contrast, bifidobacteria are usually reported as sensitive to Gram-positive spectrum antibiotics such as erythromycin, novobiocin and vancomycin, as well as to rifampicin, chloramphenicol, penicillin and ampicilin (Klare *et al.*, 2005; Ammor *et al.*, 2007; Domig *et al.*, 2007).
These observations are in accordance with our data for phenotypic susceptibility testing, where all bifidobacteria strains tested exhibited no or small (up to 12 mm) diameters of inhibition against gentamycin, neomycin, kanamycin, streptomycin, nalidixic acid and mupirocin. Because no resistance genes for these antibiotics were detected by microarray hybridization, these resistances are considered intrinsic and due to inherent properties of the strains, such as absence of the target, particular cell wall structure or metabolic properties.

Similarly, all bifidobacteria strains tested exhibited large inhibition diameters (from 23 to 45 mm) for antibiotics erythromycin (with the exception of \textit{B. longum} DSM20219: 18 mm), vancomycin, rifampicin, chloramphenicol, penicillin and ampicillin. Only for novobiocin, intermediate diameters of inhibition of 13 to 20 mm were observed.

In contrast, behavior of strains tested in this work towards tetracycline was variable, reflecting the general situation in bifidobacteria (Masco \textit{et al.}, 2006; Delgado \textit{et al.}, 2005). Indeed, the three \textit{Bifidobacterium} faecal isolates RBL67, RBL68 and RBL70, as well as \textit{B. thermophilum} DSM20210 showed diameter of inhibition of at most 15 mm, which were comparable to \textit{B. lactis} DSM10140 (19 mm), whereas \textit{B. longum} DSM20219, \textit{B. pseudolongum} DSM20092 and \textit{B. animalis} DSM20104 showed sensibility to tetracycline with inhibition diameters of 33, 35 and 29 mm respectively.

Data obtained with microarray analysis attributed the tetracycline resistant phenotype of strain \textit{B. thermophilum} RBL67 to the presence of the \textit{tet(W)} determinant, the most widely distributed tetracycline resistance gene found in bifidobacteria and in particular in \textit{B. thermophilum} strains (Mayrhofer \textit{et al.}, 2007; Ammor \textit{et al.}, 2007). Specific PCR amplification
confirmed presence of this gene in *B. thermophilum* RBL67. The *tet*(W) gene was also detected by PCR in all bacteria tested, except *B. pseudolongum* DSM20092. Presence of this gene in the *Bifidobacterium* faecal isolates RBL68 and RBL70 confirms data from the study of Kheadr *et al.* (2007), where these strains had minimum inhibitory concentration values of 62.5 µg/ml for tetracycline.

The presence of acquired antibiotic resistance determinant in strains intended for use as probiotic is not wanted, the risk being that beneficial bacterial populations act as reservoir of resistance genes and play a role in the transfer of antibiotic resistances to intestinal pathogenic bacteria, compromising the success of antibiotic therapies to treat infectious diseases. But until now, conjugation experiment by filter mating failed to show transferability of the *tet*(W) gene *in vitro* from *Lb. reuteri* SD2112 to *E. faecalis* JH2-2 and *Lc. lactis* Bu2-60 (Kastner *et al.*, 2006) or from *B. animalis* subsp. *lactis* LMG11615 to *B. adolescentis* LMG10734 (Masco *et al.*, 2006).

Additionally, in this work, we showed the absence of extrachromosomal element in *B. thermophilum* RBL67 and constructed genomic libraries of this strain in pFUN, which will be used for transformation in *Lc. lactis* and screening for nuclease activity, for identification of export signal regions on the genome of *B. thermophilum* RBL67. This constitutes a first step towards identification of the bacteriocin produced by this strain, which should be identified among the exported proteins, under the assumption that the signal sequence responsible for the export of the bacteriocin is able to target the nuclease for export in the heterologous host *Lc. lactis*. In addition to the identification of the bacteriocin, which would be the first bacteriocin identified produced by a *Bifidobacterium*,
analysis of protein export in *B. thermophilum* RBL67 will allow a better comprehension of the mechanisms involved in the interactions of this strain with its environment, a particularly important aspect in relation to potential probiotic properties.

### 4.6 Conclusion

Using microarray approach, we could detect the presence of the tetracycline resistance gene *tet*(W) in *B. thermophilum* RBL67, which could be an issue regarding probiotic application of this strain if its transferability is proven. Secondly, we showed the absence of plasmid DNA in *B. thermophilum* RBL67 and constructed genomic libraries of this strain in the plasmid pFUN, a first step towards identification of exported proteins, in particular the bacteriocin thermophilicin B67.
Co-cultivation of a bacteriocin-producing mixed culture of *Bifidobacterium thermophilum* RBL67 and *Pediococcus acidilactici* UVA1 isolated from baby faeces

Data presented in this chapter will be submitted for publication in the Journal of Applied Microbiology in 2008
5.1 Abstract

5.1.1 Background

Development of mixed culture strains to obtain products cumulating beneficial properties of many strains is a new trend in probiotic research. In addition, bacteriocin production is thought to play an important role in beneficial properties of strains used for probiotic application. In this work, *Pediococcus acidilactici* UVA1 and *Bifidobacterium thermophilum* RBL67, previously isolated as a consortium from human baby faeces and producing proteinaceous anti-*Listeria* compounds, were assessed during co-culture experiments for their potential application as mixed probiotic culture with antimicrobial properties.

5.1.2 Results

Cell growth, antimicrobial activity, glucose consumption, organic acid production and pediocin-gene expression were monitored during pure and mixed strain cultures performed in 500-ml Sixfors reactors with controlled pH (6.0) and temperature (37 °C) conditions. The balance of the two strains in mixed cultures was stable, yielding high cell count of $10^9$ cfu/ml after a very short incubation time of 4 hours for UVA1 and 7 hours for RBL67. Moreover, RBL67 was not affected by the production of pediocin by UVA1 during co-culture. Furthermore, a real-time PCR assay was developed and allowed gene-expression analysis of the pediocin-gene *pedA*. 
5.1.3 Conclusion

The co-culture of RBL67 and UVA1 showed high stability, cell yields and bacteriocin production during batch cultures. This is the first study reporting of a stable mixed culture of two bacteriocin-producing strains of human origin with potential for use as a probiotic mixture with antibacterial properties. In addition, a gene-expression real-time PCR assay was successfully developed, used for the relative quantitation of the pediocin transcript pedA and demonstrated to be a valuable complementary tool to activity assay.

5.2 Background

The development of probiotic strains in food and feed with the objective of promoting consumer’s health is a topic of growing interest for food biotechnology. The concept is based on the observation that gut microflora composition can be altered through bacterial intake by diet (Tuohy et al., 2007). Probiotics can be defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host beyond inherent basic nutrition (FAO/WHO, 2002). The majority of probiotic strains belongs to the genera Bifidobacterium and Lactobacillus, which are natural inhabitants of the normal human colon. Well-documented beneficial effects for probiotics include growth inhibition of pathogenic bacteria (the so-called barrier effect), increased resistance to infectious diseases in the gastrointestinal tract (GIT), reduction of lactose intolerance symptoms and modulation of the immune system (Parvez et al., 2006). Other effects, such as reduction of serum cholesterol levels, antihypertensive effect, alleviation of postmenopausal disor-
ders and certain anti-carcinogenic activity are also promisingly attributed to probiotics despite some contradictory results and although evaluation has often been performed only in vitro (Liong, 2007). The antagonistic effects of probiotic bacteria against pathogen microorganisms in vivo are due to competition for nutrients and adhesion sites on intestinal epithelial surfaces, immune simulation and/or in situ secretion of antimicrobial substances such as organic acids, hydrogen peroxide, bioactive peptides, bacteriocins or other low molecular weight compounds such as reuterin (Liong, 2007; Cleusix et al., 2007). However, scientific evidences of antimicrobial mechanisms of probiotics in the gut are still lacking.

Bacteriocins are ribosomally synthesized, extracellularly released peptides, that have bactericidal or bacteriostatic effect on other (usually related) species (Flynn et al., 2002). Production of bacteriocin is claimed to be an important characteristic of probiotics, promoting colonization of the GIT and exerting antagonistic activity against pathogens (Collado et al., 2005b; Deraz et al., 2007). Many bacteriocins produced by lactic acid bacteria have been shown to be active against a number of Gram-positive food spoilage and/or pathogenic bacteria, such as Listeria monocytogenes (Eijsink et al., 2002). The bacteriocin producing Lactobacillus johnsonii La1 is commercially used as probiotic strain and have recently been shown to inhibit Helicobacter pylori in vitro and in clinical trials (Gotteland et al., 2008). Bifidobacteria constitute an important component of the beneficial gut microflora, especially in breastfed newborns (Tuohy et al., 2005). Production of bacteriocin by bifidobacteria is a rare characteristic. To date, only bifidocin B has been described as a bacteriocin produced by B. bifidum NCFB 1454 (Yildirim and Johnson, 1998; Yildirim et al., 1999), but since its purification, no
additional data has been published on this bacteriocin. Pediococci are widely used in the food industry mainly as protective and starter cultures to avoid contamination of fermented sausages (Antara et al., 2004; Foegeding et al., 1992), or as probiotic in animal feed additives (Guerra et al., 2007; Weinberg et al., 2004). Due to their ability to produce antimicrobial compounds that are active against foodborne pathogens such as \textit{L. monocytogenes}, especially the broad range bacteriocin pediocin PA-1, application of pediococci as both probiotic and bioprotective cultures, as well as fermenting agents in meat products have been suggested (Työppönen et al., 2003).

Use of mixtures of several strains with beneficial effects is a new trend in the development of probiotic products. For this, strains with different origins and properties are separately produced and mixed in the final product. As an example, the efficacy of the probiotic mixture VSL\#3®, containing eight different probiotic strains, has been proven in randomized, placebo-controlled trials to maintain remission of inflammatory colitis (Gionchetti et al., 2000, 2003). Although the underlying mechanisms are still unknown, probiotic combinations offer the advantage of cumulating the positive effects of several strains and they are thought to perform more efficiently than single strain cultures, due to possible symbiotic or complementary effects in the gut environment.

\textit{Bifidobacterium thermophilum} RBL67 and \textit{Pediococcus acidilactici} UVA1 were isolated in a previous study as a stable consortium from infant faecal material and were both shown to produce proteinaceous anti-\textit{Listeria} compounds (von Ah et al., 2007; Touré et al., 2003). Moroni et al. (2006) observed that \textit{B. thermophilum} RBL67 was able to reduce the adherence of \textit{Listeria monocytogenes} to epithelial cell lines and to reduce invasion
of these cell lines in vitro. Zihler et al. (2007) showed that this strain was competitive in vitro in an intestinal environment and able to prevent and fight Salmonella infection in vitro. The bacteriocin produced by P. acidilactici UVA1 was recently identified as pediocin PA-1, encoded by the pedA gene (Mathys et al., 2007), while B. thermophilum RBL67 synthesised a new, highly hydrophobic, bacteriocin-like inhibitory substance (BLIS) active against Lactobacillus acidophilus and Listeria spp. (von Ah et al.). In view of developing new probiotic mixed-cultures, the combination of a pediocin-producing lactic acid bacterium and a bacteriocin producing Bifidobacterium strain, both of human intestinal origin, is of particular interest. Moreover, no data has been published yet on production of probiotic strains in mixed culture and producing bacteriocin.

The aim of this work was therefore to gain understanding of the interactions between these two bacteriocinogenic strains, a step towards development of probiotic mixture with antimicrobial features and demonstrated mechanism. We investigated growth kinetics and metabolism of the two strains during controlled batch fermentations with pure and mixed cultures, production of antimicrobial activity and pediocin gene expression.

5.3 Material and methods

5.3.1 Bacterial strains and growth conditions

Bifidobacterium thermophilum RBL67, producing a bacteriocin-like inhibitory substance (von Ah et al., 2007), and Pediococcus acidilactici UVA1, producing pediocin PA-1 (Mathys et al., 2007) were co-isolated from human faecal material (Touré et al., 2003). Pediococcus acidilactici UVA1-bac− was obtained by curing of the strain UVA1 from its plas-
mid, leading to the loss of the ability to produce pediocin (Mathys et al., 2007). *Listeria ivanovii* HPB28 was obtained from the Health Protection Branch Canada (Health and Welfare, Ottawa, Canada) and was used as indicator organism for bacteriocin activity. *Bifidobacterium* and *Pediococcus* strains were routinely grown overnight at 37 °C in MRSC medium, consisting of de Man, Rogosa and Sharpe medium (de Man et al., 1960) obtained from Biolife (Mailand, Italy) and supplemented with 0.05 % L-cysteine hydrochloride (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). *B. thermophilum* RBL67 was incubated in anaerobic jars with an atmosphere generation system (AnaeroGen TM, Oxoid, Basel, Switzerland). *L. ivanovii* HPB28 was propagated overnight at 30 °C in TSY medium consisting of tryptic soy broth (Oxoid) containing 0.6 % (w/v) yeast extract (Merck, Darmstadt, Germany). For agar plates, the media were supplemented with 1.5 % (w/v) agar (Becton Dickinson, New York, USA). Bacterial stocks were stored at -80 °C in appropriate media supplemented with 33 % (v/v) glycerol and subcultured three times at one day intervals on fresh agar plates before use.

### 5.3.2 Determination of selective conditions for RBL67 cell enumeration

Nine potential bifidobacteria-specific media were tested for their ability to allow discriminative growth of *B. thermophilum* RBL67 but not *P. acidilactici* UVA1 (Table 5.1). Overnight grown cultures of *B. thermophilum* RBL67 and *P. acidilactici* UVA1 (at 37 °C in 10 ml MRSC) were either used pure or mixed in a 1:1 ratio and serially 10-fold diluted in reduced saline solution (8.5 g/l NaCl, 1 g/l peptone, 0.05 % L-cysteine hydrochloride, pH 6-7). Aliquots of 0.1 ml of appropriate dilutions were
plated on each selective medium and incubated for 48 h at 37 °C before cell count determination. To test electivity, pure cultures were plated in parallel on MRSC and incubated anaerobically for RBL67 and aerobically for UVA1. Randomly picked colonies were observed microscopically for confirmation of the rod- or coccoid- shape.

5.3.3 Culture experiments in bioreactors

Single culture and co-culture fermentations were performed in 500-ml Sixfors bioreactors (Infors AG, Bottmingen, Switzerland) in a total volume of 400 ml MRSC medium with stirring by flat blade impeller at 150 rpm. Fermentations were carried out for 24 h at 37 °C and pH 6.0, maintained with 5 M NaOH. For pure cultures, the medium was inoculated either with 5 % (v/v) of an overnight culture of \textit{B. thermophilum} RBL67 or with 1 % (v/v) of \textit{P. acidilactici} UVA1 or UVA1-bac\textsuperscript{−}. For mixed cultures, overnight cultures were used to inoculate the bioreactors with 5 % (v/v) of \textit{B. thermophilum} RBL67 and 0.5 % (v/v) of either \textit{P. acidilactici} UVA1 or UVA1-bac\textsuperscript{−}. \textit{B. thermophilum} RBL67 was inoculated at a higher concentration than \textit{P. acidilactici} to compensate for its longer lag phase. Each fermentation was carried out twice. Samples were withdrawn aseptically from the bioreactor every 50 min during the exponential growth phase and every hour afterwards until 12 h, with a last sample after 24 h. Monitoring of growth was performed by optical density measurement at 600 nm (OD\textsubscript{600}) against sterile medium with a Uvikon 810P photometer (Kontron Instruments, Rotkreuz, Switzerland). Fermentation samples were 10-fold diluted with sterile MRSC medium when OD\textsubscript{600} was higher than 0.6. At each sampling point, 0.1 ml broth was taken for determination of colony forming units (cfu/ml), 0.5 ml
5 Characterisation of the mixed culture of strains RBL67 and UVA1

Table 5.1: Potential media for selective growth of *B. thermophilum* RBL67

<table>
<thead>
<tr>
<th>Medium</th>
<th>Selective agent(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raffinose-Bifidobacterium</td>
<td>0.05 % cysteine</td>
<td>Hartemink <em>et al.</em> (1996)</td>
</tr>
<tr>
<td></td>
<td>3 g/l lithium chloride</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 g/l Na-propionate</td>
<td>Dave and Shah (1996)</td>
</tr>
<tr>
<td>MRSC-NNLP</td>
<td>0.05 % cysteine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 mg/l nalidixic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 mg/l neomycin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 g/l lithium chloride</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 mg/l paromomycin sulfate</td>
<td></td>
</tr>
<tr>
<td>Beerens</td>
<td>0.05 % cysteine</td>
<td>Beerens (1990)</td>
</tr>
<tr>
<td></td>
<td>0.5 % propionic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 5</td>
<td></td>
</tr>
<tr>
<td>Beerens modified</td>
<td>0.05 % cysteine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 g/l Na-propionate</td>
<td></td>
</tr>
<tr>
<td>Lapierre modified</td>
<td>3 g/l Na-propionate</td>
<td>Lapierre <em>et al.</em> (1992)</td>
</tr>
<tr>
<td></td>
<td>2 g/l lithium chloride</td>
<td></td>
</tr>
<tr>
<td>Bifidus selective medium</td>
<td>Sigma-Aldrich, Buchs, Switzerland</td>
<td></td>
</tr>
<tr>
<td>mMRSC</td>
<td>0.05 % cysteine</td>
<td>Rada <em>et al.</em> (1999)</td>
</tr>
<tr>
<td></td>
<td>5 g/l mupirocin</td>
<td></td>
</tr>
<tr>
<td>mWilkins-Chalgren</td>
<td>5 g/l mupirocin</td>
<td></td>
</tr>
</tbody>
</table>
5.3 Material and methods

for metabolic activity measurement, 0.5 ml for anti-Listeria activity determination and sample volumes corresponding to approximately $5 \times 10^8$ cells were harvested for RNA extraction and quantification of pediocin transcript.

5.3.4 Viable cell enumeration and growth rate calculation

Culture samples (0.1 ml) were serially diluted in saline solution and appropriate dilutions were plated on adequate agar medium and incubated for 48 hours at 37°C. For single cultures, all strains were plated on MRSC and incubation was done anaerobically for *B. thermophilum* RBL67 and aerobically for pediococci. For mixed cultures, selective conditions were used with anaerobic incubation on mMRSC agar, consisting of MRSC supplemented with 5 g/l mupirocin, for *B. thermophilum* RBL67 and aerobic incubation on MRSC for pediococci. Each dilution was plated twice. The maximum specific growth rate of a strain during batch culture was calculated by linear regression analysis on data from the natural logarithm of cell counts as a function of time during the exponential growth phase. Results are expressed as means and standard deviations for two repeated fermentations.

5.3.5 Metabolic activity measurement

Glucose, acetate and lactate concentrations were measured in duplicate by HPLC analysis (Merck Hitachi, San Jose, CA, USA) using an Aminex HPX-87H ion exchanger column (BioRad, Hercules, CA, USA). Culture samples were centrifuged 5 min at 13’000 x g and supernatants diluted 5 fold in HPLC-grade water and filtrated through 0.45 µm pore size filters before analysis. Glucose and organic acids were measured by refractive
index detector. The mobile phase was 0.01 M H$_2$SO$_4$ at a flow rate of 0.6 ml/min. Calculation of the concentration was based on calibration curves prepared with pure standards.

### 5.3.6 Inhibition assays with culture supernatants

Broth samples (0.5 ml) were centrifuged for 5 min at 13'000 x g and cells were discarded. The supernatant was heated for 10 min at 95 °C and stored overnight at 4 °C. Total anti-Listeria activity was measured turbidimetrically using a critical dilution micro-method (Turcotte et al., 2004), with *L. ivanovii* HPB28 as indicator organism. Twofold serial dilutions of samples were performed in 0.125 ml of TSY buffered with 0.2 % (w/v) sodium hydrogen carbonate, in a microtiter plate (Orange Scientific, Braine-l’Alleud, Belgium). Finally, 50 µl of an overnight culture, diluted 10'000 times, of the indicator strain *L. ivanovii* HPB28, containing approximately 1.5x10$^4$ cfu, were dispensed into each well. The microtiter plate was incubated at 30 °C for 18 h before reading the optical density at 590 nm (FL600 microplate fluorescence reader, Bio-Tek, Winooski, USA). One arbitrary unit (AU) was defined as a 0.125-ml portion of the highest dilution of bacteriocin preparation that inhibited growth in a well after 18 h. Inhibition was measured when optical density in a well was less than half the optical density of a control well with fresh medium replacing the bacteriocin sample. Activity of a sample was expressed in arbitrary units per ml (AU/ml), calculated with the formula AU/ml = (1’000/125)*2$^N$, where N stands for the number of inhibited wells. Each sample was analysed in duplicate. Measurements were highly reproducible for the repeated fermentations and differed by no more than one well, corresponding to a twofold dilution.
5.3.7 RNA isolation and reverse transcription

Immediately after sampling, cells were harvested by centrifugation for 1 min at 4000 x g, snap-frozen in liquid nitrogen and stored at -70 °C until RNA extraction. RNA from *P. acidilactici* UVA1 and UVA1-bac− was isolated with the RNeasy Mini kit (Qiagen, Basel, Switzerland), with the following modification: cell lysis was performed for 20 min at 37 °C in presence of 3 mg lysozyme and 20 U mutanolysine and the mixture was vigorously mixed by vortexing for 5 min. Total extracted RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and quantified by measurement of absorption at 260 nm (Uvikon 810P photometer). Amounts of 500 ng or 1 µg exactly were used for cDNA synthesis with the Omniscript reverse transcription kit (Qiagen) according to supplier’s instruction and in the presence of 10 U RNase inhibitor (RNaseOUT, Invitrogen, Carlsbad, CA, USA).

5.3.8 Development and validation of a pedA-qPCR assay

Oligonucleotide primers and probes used in this study were purchased from Microsynth (Balgach, Switzerland) and are presented in Table A.1. TaqMan probes were labeled with the fluorescent reporter dye FAM (6-carboxyfluorescin) at the 5’-end and the quenching group TAMRA (6-carboxytetramethylrhodamine) at the 3’-end. For amplification and relative quantification of the pedA transcript, primers pedA2RTF and pedA2RTR, as well as the TaqMan probe TqMpedA were designed with the program PrimerExpress 1.5. As an endogenous control the single copy gene tufA was chosen. A 0.8-kb fragment was first amplified from strain UVA1 with degenerate primers U1 and U3 originally designed for
5 Characterisation of the mixed culture of strains RBL67 and UVA1

tufA in enterococci (Ke et al., 2000). The fragment was sequenced [GenBank: EF623895] and primers tufARTF and tufARTR as well as the TaqMan probe TqMtufA were designed based on this sequence. Validation of the system was performed according to tutorials published by Applied Biosystems (http://www.appliedbiosystems.com, November 2007). The linear dynamic range and PCR efficiency (E) for each target gene were determined by generating cDNA dilution curves. Known amounts of cDNA, ranging from 0.26 pg to 100 ng, were subjected to real-time PCR analysis. The threshold cycle (C_T; the number of cycles at which fluorescent emission first exceeded the baseline value) for each cDNA amount was plotted against the log of cDNA concentration. The slope of this curve was determined and PCR efficiency was calculated as follow: E = (10^{(-1/slope)}−1)∗100. The efficiencies of amplification of the two systems were compared by plotting the ΔC_T (C_T\_pedA- C_T\_tufA) against the log of input cDNA quantity.

5.3.9 Relative quantification of the pedA transcript by real-time PCR

For relative quantification of pediocin-gene expression by real-time PCR, cDNA quantities corresponding to 10 ng of RNA, in a volume of 5 µl, were used as template. Reactions were performed in triplicate in 25 µl containing 0.3 µM of each primer, 0.2 µM of TaqMan probe and 12.5 µl of 2x qPCR MasterMix (Eurogentec, Seraing, Belgium). Amplification conditions were as follows: 2 min at 50 °C, HotGoldStart activation for 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. ROX was used as internal passive reference dye. For relative quantification, the comparative C_T method was used. The threshold cycle (C_T) values
for the target gene \( \text{pedA} \) and the reference gene \( \text{tufA} \) were measured and differences between the two C\( _T \) values (\( \Delta \text{C}_T \)) were calculated. The \( \Delta \text{C}_T \) values of each time point during the fermentation were finally compared to the \( \Delta \text{C}_T \) value for the reference sample taken 1 h after inoculation of the reactor (\( \Delta \Delta \text{C}_T \)). Results were expressed as fold-differences (\( 2^{-\Delta \Delta \text{C}_T} \)) in \( \text{pedA} \) transcript quantities at each time point relative to the reference sample. The samples were subjected to electrophoresis on 2 % agarose gels which were stained with ethidium bromide to confirm the presence of the expected DNA products and the absence of unwanted non-specific amplicons.

5.3.10 Statistical analysis

Values for specific growth rate, maximal cell production and relative \( \text{pedA} \) expression are means and standard deviations from two independent repetitions. Data for maximal cell production were normalized by a \( \log_{10} \) transformation. Differences between means were tested for statistical significance using the t-test. Statistical significance was estimated at the probability level \( p<0.05 \).

5.4 Results

5.4.1 Selective media for enumeration of RBL67 cells from mixed cultures

Specific cell enumeration of \( B. \ thermophilum \) RBL67 and \( P. \ acidilactici \) UVA1 in mixed cultures required selective growth conditions for each strain. Serial plating of pure cultures on selective agars presented in Table 5.1 showed that four of the nine media tested allowed selective growth
of *B. thermophilum* RBL67 under anaerobic conditions whereas *P. acidilactici* UVA1 did not grow: mWilkins-Chalgren, Beerens, mMRS and BSM. The latter three were tested for their ability to allow selective growth of *B. thermophilum* RBL67 from mixed samples. Total absence of growth of *P. acidilactici* UVA1 was only observed on mMRS, whereas growth of *B. thermophilum* RBL67 was not influenced by the presence of mupirocin, yielding same counts of colony forming units on mMRS than on the non-selective MRSC. Therefore, MRSC supplemented with mupirocin was used for cell number determination of *B. thermophilum* RBL67 from co-culture samples, being elective for this strain and effectively selective against *P. acidilactici*.

### 5.4.2 Cell growth during single and mixed cultures

Growth profiles of *Pediococcus* strains UVA1 and UVA1-bac− in pure cultures were similar and characterized by fast growth, with exponential growth until 4 h of fermentation followed by short transition phase and stationary phase starting after 6 h culture (Figure 5.1). Maximum specific growth rates did not differ significantly in single- or mixed-cultures (Table 5.2), whereas maximal cell production was significantly lower for *P. acidilactici* UVA1-bac− in mixed cultures with *B. thermophilum* RBL67 compared to single cultures (p=0.01, Table 5.2).

For *B. thermophilum* RBL67, maximum specific growth rates and cell production were not statistically different for single and mixed cultures (Table 5.2), although cell counts of $10^9$ cfu/ml were reached quicker during mixed- (after only 7 h of fermentation) than during single cultures (after 12 h of fermentation).

Furthermore, growth curves profiles of *B. thermophilum* RBL67 showed
5.4 Results

differences for pure and mixed cultures. During pure cultures, cell counts increased regularly to reach $1.1 \times 10^9 \pm 0.3 \times 10^9$ cfu/ml after 12 h and remained stable until 24 h. For mixed cultures with *P. acidilactici* UVA1 or UVA1-bac$^{-}$, cell counts reached a maximum after 9 and 11 h, respectively, (Table 5.2) and then decreased to $3.4 \times 10^8 \pm 0.6 \times 10^8$ and $5.8 \times 10^8 \pm 4.0 \times 10^8$ cfu/ml, respectively, after 24 h (Figure 5.2).

Table 5.2: Maximum specific growth rates and cell production during pure and mixed strain cultures of *B. thermophilum* RBL67, *P. acidilactici* UVA1 and *P. acidilactici* UVA1-bac$^{-}$. In parenthesis are the p-values for the t-test comparing data of mixed cultures with single cultures (with two repetitions of each fermentation).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Single or mixed culture</th>
<th>Specific growth rate ($\mu$) (h$^{-1}$)</th>
<th>Maximal cell production ($x 10^9$ cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBL67</td>
<td>in single culture</td>
<td>0.33 $\pm$ 0.01</td>
<td>1.1 $\pm$ 0.3</td>
</tr>
<tr>
<td></td>
<td>in co-culture with UVA1</td>
<td>0.43 $\pm$ 0.06 (0.95)</td>
<td>1.4 $\pm$ 0.0 (0.43)</td>
</tr>
<tr>
<td></td>
<td>in co-culture with bac$^{-}$</td>
<td>0.29 $\pm$ 0.03 (0.71)</td>
<td>1.6 $\pm$ 0.9 (0.75)</td>
</tr>
<tr>
<td>UVA1</td>
<td>in single culture</td>
<td>1.29 $\pm$ 0.04</td>
<td>4.0 $\pm$ 0.8</td>
</tr>
<tr>
<td></td>
<td>in co-culture with RBL67</td>
<td>1.41 $\pm$ 0.01 (0.36)</td>
<td>2.0 $\pm$ 0.3 (0.12)</td>
</tr>
<tr>
<td>UVA1-bac$^{-}$</td>
<td>in single culture</td>
<td>1.20 $\pm$ 0.08</td>
<td>6.4 $\pm$ 0.4</td>
</tr>
<tr>
<td></td>
<td>in co-culture with RBL67</td>
<td>1.35 $\pm$ 0.11 (0.28)</td>
<td>2.1 $\pm$ 0.1 (0.01)</td>
</tr>
</tbody>
</table>
5 Characterisation of the mixed culture of strains RBL67 and UVA1

Figure 5.1: Growth and metabolic activity of *Pediococcus acidilactici* UVA1 and UVA1-bac<sup>−</sup> during single and mixed cultures, with: (a) *P. acidilactici* UVA1, (b) *P. acidilactici* UVA1 and *B. thermophilum* RBL67, (c) *P. acidilactici* UVA1-bac<sup>−</sup> and (d) *P. acidilactici* UVA1-bac<sup>−</sup> and *B. thermophilum* RBL67. Viable cell counts (full diamonds), glucose (empty squares), lactate (empty triangles) and acetate (empty circles) concentrations. Values are average and standard deviations of two repetitions.
Figure 5.2: Growth and metabolic activity of *B. thermophilum* RBL67 during single and mixed culture, with: (a) *B. thermophilum* RBL67, (b) *B. thermophilum* RBL67 and *P. acidilactici* UVA1 and (c) *B. thermophilum* and *P. acidilactici* UVA1-bac−. Viable cell counts (full diamonds), glucose (empty squares), lactate (empty triangles) and acetate (empty circles) concentrations. Values are average and standard deviations of two repetitions.
5.4.3 Metabolic activity during single and mixed cultures

During single cultures of *P. acidilactici* UVA1 or *P. acidilactici* UVA1-bac\(^{-}\), glucose was totally consumed after 11 h, which corresponded to the stop of lactate production. Lactate concentration in the fermentation medium increased during this time from 0.5 ± 0.1 g/l to 14.5 ± 0.1 g/l and remained unchanged until 24 h. Concentration of acetate was stable at 3.7 ± 0.1 g/l during 24 h culture. During single cultures of *B. thermophilum* RBL67, glucose depletion occurred after 11 h, corresponding to the stop of lactate and acetate productions. Lactate concentration in the fermentation medium increased during this period from 0.6 ± 0.0 to 6.0 ± 0.1 g/l and acetate from 3.8 ± 0.1 to 10.2 ± 0.1 g/l. These values remained stable from 11 h to the end of the fermentation. During mixed cultures of *B. thermophilum* RBL67 and *P. acidilactici* UVA1 or *P. acidilactici* UVA1-bac\(^{-}\), glucose concentration dropped from 14.3 ± 0.2 g/l to undetectable concentration in only 9 h, corresponding to the stop of lactate and acetate production. Lactate concentration increased during this time from 0.6 ± 0.0 to 10.5 ± 0.0 g/l and acetate from 3.9 ± 0.0 to 7.6 ± 0.1 g/l, corresponding to a total metabolite production of approximately 13.6 g/l.

5.4.4 Antimicrobial activity during single and mixed cultures

Antimicrobial activity measured with a microtiter plate assay and *L. ivanovii* HPB28 as indicator strain during single and mixed cultures are presented in Figure 5.3. No activity was detected with this method in fermented broth of pure cultures of *B. thermophilum* RBL67. For pure cultures of *P. acidilactici* UVA1, activity increased from 96 AU/ml at
the beginning of the fermentation to 3072 AU/ml after 8 h, remained constant until 12 h and decreased thereafter to 1536 AU/ml after 24 h. A similar profile was measured with the mixed cultures of *P. acidilactici* UVA1 and *B. thermophilum* RBL67, but with a lower maximum activity level of 1024 AU/ml after 7 h and a very large decrease of activity to 80 AU/ml measured after 24 h. No activity was tested for the pure and mixed cultures of *P. acidilactici* UVA1-bac<sup>−</sup>.

![Graph](image)

**Figure 5.3:** Anti-*Listeria* activity tested in the fermentation medium pure (circles) or mixed (triangles) cultures of *P. acidilactici* UVA1 with *B. thermophilum* RBL67. Activity data from repeated cultures differed by no more than one well, corresponding to a twofold dilution.
5 Characterisation of the mixed culture of strains RBL67 and UVA1

5.4.5 Validation of the qPCR assay

RNA extraction, cDNA synthesis and real-time PCR analysis of samples taken at different time points during growth of *P. acidilactici* UVA1 showed that *tufA* was expressed at a constant rate until early stationary phase (corresponding to a fermentation time of 8 h), with a CT value of 16.4 ± 0.3 (data not shown). Amplification efficiencies of both *pedA* and *tufA* transcripts with the designed primers were determined on a serial 5-fold dilution of one cDNA sample synthesized from RNA isolated during the exponential growth phase. High amplification efficiencies of 99.03 % ($R^2 = 0.999$) and 98.76 % ($R^2 = 0.999$), were measured for *pedA* and *tufA* transcripts, respectively, for template-cDNA quantities ranging from 0.26 pg to 100 ng (corresponding to 5 log units). Within this range, the amplification efficiencies were comparable for the two systems.

5.4.6 Pediocin-transcript production during co-culture

RNA extraction and real-time PCR were used to analyse the relative pediocin-gene expression of *P. acidilactici* UVA1 during the first eight hours of pure or mixed cultures with *B. thermophilum* RBL67. For both, the *pedA*-gene was constantly expressed during exponential growth phase (between 1 and 3 h culture) whereas an increase of the relative *pedA* expression was observed at the end of the exponential growth phase. Expression was high and stable during transition and early stationary growth phase, between 5 and 8 h (Figure 5.4). Although expression seemed to occur at lower level during pure culture of *P. acidilactici* UVA1 than during mixed culture, the difference was not statistically significant due to important standard deviations (Figure 5.4).
Figure 5.4: Relative pedA gene expression measured with real-time PCR during single (circles) and mixed (triangles) culture of *P. acidilactici* UVA1 with *B. thermophilum* RBL67. Values are means for two independent cultures and are expressed as fold-differences ($2^{-\Delta\Delta C_T}$) in pedA transcript quantities relative to *tufA* at each time point relative to the start of the fermentation. Bars indicate standard deviations for 2 repeated cultures with three replicates for each sample.
5.5 Discussion

*P. acidilactici* UVA1 and *B. thermophilum* RBL67 both originate from the human intestine and produce antimicrobial peptides active against *Listeria* spp. (Mathys *et al.*, 2007; von Ah *et al.*, 2007), two properties that suggest potential for probiotic application. In this work, we demonstrated a further promising property of these two strains: their ability to grow in co-culture, both strains reaching high maximal cell counts, similar to those measured during pure cultures, and high anti-*Listeria* activity.

Our data indicate that *B. thermophilum* RBL67 is a competitive strain in co-culture with *P. acidilactici* UVA1. Indeed, neither the presence of fast growing pediococci, nor the production of pediocin limited the growth of RBL67 during co-culture. These results are in accordance with the work of Kheadr *et al.* (2004) and Le Blay *et al.* (2007), who tested *in vitro* the sensitivity to pediocin PA-1 of several intestinal *Bifidobacterium* isolated from infants, among which *B. thermophilum* RBL67, as well as four Gram-negative and 17 Gram-positive common intestinal and probiotic bacterial strains. They observed no inhibition of this bacteriocin on intestinal strains in contrast to nisin A and Z.

Total anti-*Listeria* activity measured during pure and mixed strain batch cultures of *P. acidilactici* UVA1 and *B. thermophilum* RBL67 increased in correlation with cell growth, which was not different for both cultures. It is important to note that both UVA1 and RBL67 produce antimicrobial compounds active against *L. ivanovii* but the activity assay used in this study does not allow discrimination of antimicrobial activities from each strain. Contribution of *B. thermophilum* RBL67 to total
anti-
*Listeria* activity during mixed culture was likely very small as observed previously by von Ah *et al.* (2007). In that study, bacteriocin activity during batch cultures of *B. thermophilum* RBL67 with optimal conditions could only be detected after a ten-fold concentration of the samples. In our study, a gene expression assay was developed to measure specifically expression of the pediocin during single or mixed cultures.

For such an assay, reliable relative quantification of the target transcript is dependant on the detection of an internal reference transcript which is constantly expressed in all samples, allowing normalisation of data (Livak and Schmittgen, 2001). We successfully used *tufA*, encoding a translational elongation factor as endogenous control. Its expression was constant during the exponential growth phase and until the early stationary phase. Based on this observation, a pediocin-gene expression assay was developed and used to follow pediocin transcript production during exponential growth phase of *P. acidilactici* UVA1 during single and mixed cultures.

We observed that expression of the pediocin gene was not significantly affected by the presence of *B. thermophilum* RBL67 in co-cultures compared to single cultures. Expression of the *pedA* transcript was constant during the exponential phase, increased at the end of this growth phase and remained constant at a higher level during the transition and early stationary phases. The constant expression of *pedA* relative to *tufA* during exponential growth phase is consistent with the fact that, in contrast to nisin production by lactococci, no self-regulatory element was found upstream of the pediocin operon in *P. acidilactici* UVA1 (Mathys *et al.*, 2007). This indicates that, as shown with many described bacteriocins, pediocin expression in *P. acidilactici* UVA1 is growth dependant, which
is also consequent with anti-Listeria activities measured during batch cultures. In contrast, the increased expression on the pedA gene observed at the beginning of the stationary phase does not correlate with the results obtained for pediocin activity. These data, together with the large standard deviations observed for pedA relative gene expression after 5 h of culture indicate that the real-time PCR method used here is only reliable for exponentially growing cells. This could be explained by increased degradation of the protein at the beginning of the stationary phase or impairment of the maturation of the pre-pediocin.

The pediocin-gene expression assay presented here could be adapted to follow bacteriocin-gene expression in complex systems such as food products or to assess the influence of food production conditions. Ramiah et al. (2007) recently measured expression under different conditions of the bacteriocin gene plaA of L. plantarum with a similar assay using the glyceraldehyde-3-phosphate gene as reference transcript.

A new trend in probiotic research is the application of mixed cultures to obtain products cumulating beneficial properties of many strains (Collado et al., 2007). Most of the time, the strategy consist in mixing together strains of different origins, produced in separate processes, and optimally showing different but complementary health-promoting characteristics. The co-culture of P. acidilactici UVA1 and B. thermophilum RBL67 presents additional benefits with both strains producing bacteriocin. Furthermore, both strains being of human origin and isolated as a consortium from human children faeces (Touré et al., 2003; von Ah et al., 2007), they could have high ability to co-exist in the gastrointestinal tract. Indeed, our previous observation that this co-culture was very stable upon sub-culturing and data from this study indicate that this natural strain
combination can be propagated in a single batch culture, giving a stable balance of the two strains.

5.6 Conclusion

*B. thermophilum* RBL67 and *P. acidilactici* UVA1, producing an unknown bacteriocin and pediocin PA-1, respectively, grow in stable balance during pH- and temperature-controlled batch cultures. *B. thermophilum* RBL67 was not affected by the presence of *P. acidilactici* and pediocin production. Our data suggest that the mixed culture of *P. acidilactici* UVA1 and *B. thermophilum* RBL67 has potential for application as mixed probiotic preparation with high antimicrobial feature. Furthermore, the developed real-time PCR gene-expression assay allowed reliable relative quantification of the pediocin transcript *pedA* during exponential growth phase and could be adapted for the monitoring of pediocin expression in diverse complex samples such as food or feed products.
6 General conclusions and outlooks

The hypothesis of this study was that *Bifidobacterium thermophilum* RBL67 and *Pediococcus acidilactici* UVA1 have potential for probiotic application as a mixed strains preparation. This hypothesis was formulated based on the observations that both strains produce a different bacteriocin with different activity spectra, that they both are of human origin and were isolated together as a consortium. Furthermore, *B. thermophilum* RBL67 has been previously shown *in vitro* to have probiotic properties such as reduction of adhesion of *Listeria monocytogenes* to epithelial cells and ability to compete in human intestinal environment.

In this work, molecular genetic tools were developed and applied to verify this hypothesis. More specifically, we report the development and application of molecular tools for the identification of both bacteriocins, for bacteriocin gene-expression study during mixed culture production and for the investigation of distribution of the pediocin-encoding gene pedA and of *B. thermophilum* strains in human faeces.

With this approach, we demonstrated the following characteristics for *P. acidilactici* UVA1 and *B. thermophilum* RBL67 which support our hypothesis:

Using biochemical methods in parallel to gene expression and sequencing analysis, we identified the bacteriocin produced by *P. acidilactici* UVA1 as pediocin AcH/PA-1, a proteinaceous compound with strong
anti-	extit{Listeria} activity, which was previously shown to inhibit the growth of pathogenic microorganisms without affecting commensal intestinal bacteria.

With the help of a newly developed pediocin gene specific real-time PCR assay, we showed the large distribution of the pediocin genetic determinant in human baby faecal samples.

We confirmed, by detection using a new specific real-time PCR assay and isolation of a 	extit{B. thermophilum} isolate, the presence of this species as natural inhabitant of the human gastrointestinal tract.

We showed, during mixed batch-culture experiments, that 	extit{P. acidilactici} UVA1 and 	extit{B. thermophilum} RBL67 are stable in mixed culture, both strains reaching high cell counts. Furthermore, the co-culture retains high anti-	extit{Listeria} activity. In addition, we successfully developed and used a real-time PCR assay for the relative quantification of the pediocin gene expression, which allowed measurement of transcription of the 	extit{pedA} gene during culture of 	extit{P. acidilactici} UVA1 pure or as mixed culture with 	extit{B. thermophilum} RBL67.

In addition, the real-time PCR tools developed in this work have potential for various applications. The pediocin gene expression system can be adapted for quantification of pediocin expression in food products supplemented with pediocin-producing strains or in the human gut following consumption of such products, whereas the specific 	extit{B. thermophilum} real-time PCR assay will contribute to a better knowledge of the composition of the bifidobacterial community of the human intestinal microflora, in relation to health or disease and consumption of pre- or probiotics.

However, for application of this strain combination as probiotic in food
products, the bacteriocin produced by *B. thermophilum* RBL67 has to be identified and its biochemical properties and activity spectrum characterized. In this work, a genomic library of this strain was constructed in the vector pFUN, as a first step towards identification of the exported proteins, among which the bacteriocin. Availability of its encoding sequence will allow development of a gene expression assay to follow bacteriocin gene expression in the mixed culture. In addition, the non transferability of the tet(W) determinant we identified on the genome of *B. thermophilum* RBL67 will have to be demonstrated, in order to avoid that consumption of this probiotic strain be associated with maintenance of a reservoir of antibiotic resistance genes within the human gastrointestinal tract.

To conclude, we showed that strains *P. acidilactici* UVA1 and *B. thermophilum* RBL67 exhibit characteristics conferring great potential for application as probiotic mixture with strong antimicrobial activity. Moreover, the real-time PCR assays we designed will contribute to accurate characterization of the composition of the human intestinal microflora and assessment of probiotic efficacy.
A Oligonucleotides used in this study
Table A.1: Oligonucleotides primers and probes used in this study

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<th>Primer/probe</th>
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<th>Target/purpose</th>
<th>Fragment length</th>
<th>Reference</th>
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<td>P1</td>
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<td>P2</td>
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<td>pedopR</td>
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<tr>
<td>pedseqA</td>
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<td>pedseqB</td>
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1FAM (6-carboxyfluorescine): fluorescent reporter dye, TAMRA (6-carboxytetramethylrhodamine): quencher.
Table A.1 ... continued

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1FAM (6-carboxyfluorescein): fluorescent reporter dye, TAMRA (6-carboxytetramethylrhodamine): quencher.
### Sequencing of the *B. thermophilum* RBL67 16S rDNA and specific qPCR assay

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<td>520F</td>
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<td>btherm</td>
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<td>bthermRTF</td>
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1FAM (6-carboxyfluorescine): fluorescent reporter dye, TAMRA (6-carboxytetramethylrhodamine): quencher.
Table A.1 ... continued

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1FAM (6-carboxyfluorescine): fluorescent reporter dye, TAMRA (6-carboxytetramethylrhodamine): quencher.
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Bibliography


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