DEVELOPMENT, OPTIMIZATION AND SCALE UP OF THE PRODUCTION AND PURIFICATION PROCESS OF BACTERIOCINS FROM LACTIC ACID BACTERIA

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Alla mia famiglia e a Fulvia

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ABSTRACT

Bacteriocins from lactic acid bacteria are small peptides that show antimicrobial activity towards other bacteria, generally strains closely related to the producer. As target microorganisms include pathogens and many bacteria responsible of the food spoilage, the interest in bacteriocins as food biopreservatives is greatly increased in recent years.
Three lactic acid bacteria strains were screened to evaluate their ability to produce bacteriocins active towards pathogens and, therefore, potentially interesting in food industry. One of them (*Enterococcus casseliflavus* CGA1), industrial strain isolated by "salami" processing, was able to produce a small enterocin with strong activity versus *Listeria monocytogenes*, a pathogen that causes listeriosis. In the prospect of a cost effective application of this peptide in food industry, a biotechnological process consisting of a fed-batch fermentation strategy and a purification procedure based on membranes was developed and scaled up.
First, in order to satisfy economical constraints for the industrial implementation, a growth medium suitable for microorganism growth and enterocin production was optimized. Particularly, the best complex nitrogen nutrient was identified and the concentrations of carbon and nitrogen sources were decreased evaluating the effect on bacteriocin activity. The optimized medium was used to grow the microorganism in batch fermentations. Growth parameters were properly identified through the characterization of the growth curve and the kinetics of glucose consumption and lactic acid production. The kinetics of bacteriocin production was also defined and compared with the growth curve, showing that the enterocin is a growth-related product.
Due to this, the biomass production and, therefore, the bacteriocin activity was improved through exploiting modern technologies of high cell density fermentations. Different fed-batch strategies were tried. Particularly, batch data of yield and glucose consumption were used to implement a fed-batch profile based on continuous feeding of substrate into bioreactor. This process allowed to improve about eight fold both biomass concentration and enterocin production. It was also scaled up to a pilot plant of 100 L, showing high reproducibility in terms of microorganism growth and bacteriocin titer.
In order to purify the product from the fermentation broth, an innovative downstream processing based on two ultrafiltration steps was developed and compared with traditional procedures, using large amounts of organic solvents. Membrane processes allowed to obtain the best product yield and purification fold. A spectrum activity of the purified product, comprising starter cultures and pathogens, was also established. Product was active towards dairy starter cultures, but not towards the ones used in meat processing. The results obtained seem to confirm the effective applicability of the product as natural additive to improve meat microbiological safety.
The same indicator strains were used to assay the antimicrobial activity of two lactobacilli strains, *Lactobacillus plantarum* DSM 12028 and *Lactobacillus crispatus* L1. Both strains were able to inhibit pathogenic bacteria, as *Staphilococcus aureus* and *Streptococcus agalactiae*. Therefore, they seem to be good candidates for the production of bacteriocins of biotechnological interest. The effect of different concentrations of complex nitrogen source on bacteriocin production was studied in flask experiments, as well as batch fermentations were performed in order to enhance bacteriocin activity. Particularly for *L. plantarum* DSM 12028, the controlled fermentation environment allowed to improve significantly the production of the metabolite of interest.

RIASSUNTO

Negli ultimi anni si è assistito ad un crescente sforzo finalizzato alla ricerca di nuovi prodotti deputati alla protezione degli alimenti dalla contaminazione microbica. Attualmente esiste in commercio una grande varietà di prodotti chimici con proprietà preservanti ampiamente utilizzata nell'industria per la conservazione degli alimenti. Nonostante la conservazione con sostanze chimiche di sintesi fornisca molti vantaggi, i consumatori sono sempre più attenti ai loro eventuali effetti tossici. Per tale motivo, i produttori si stanno sempre più indirizzando verso la ricerca di sostanze additive alternative, possibilmente di origine naturale, per ridurre o evitare del tutto i conservanti chimici tradizionali. Non è un caso, dunque, che negli ultimi anni grande attenzione si sta rivolgendo alla messa a punto di svariati approcci di conservazione biologica degli alimenti.

Per definizione, essa si riferisce all'utilizzo di microrganismi antagonisti (colture protettive) o dei loro prodotti metabolici (biopreservanti) per inibire o distruggere microrganismi indesiderati negli alimenti, pertanto aumentandone la sicurezza microbiologica ed estendendone la *shelflife*. La maggior parte dei moderni approcci di conservazione biologica prevedono l'utilizzo mirato e consapevole di batteri lattici o dei loro prodotti metabolici. Essi sono stati infatti utilizzati per secoli nella fermentazione e processazione di svariati alimenti, dai prodotti caseari alle carni, dai cereali ai prodotti vegetali. In questi alimenti, essi producono una grande varietà di composti che contribuiscono alla determinazione dell'aroma, colore e consistenza. Per questa loro associazione con il cibo e la lunga tradizione di batteri *food-grade*, molti di essi posseggono lo status di microrganismi GRAS (*Generally Regarded As Safe*). Negli alimenti in cui sono presenti, inoltre, essi forniscono anche un grande effetto preservante, dovuto alla capacità di produrre un vero e proprio arsenale di composti ad attività antimicrobica. Questi comprendono metaboliti a basso peso molecolare come anidride carbonica, perossido d'idrogeno e diacetile; acidi organici come acido lattico ed acido acetico; enzimi come lisozima e lattoperossidasi e, soprattutto, batteriocine.

Le batteriocine prodotte da batteri lattici possono essere definite come peptidi o piccole proteine, sintetizzate per via ribosomale e secrete nel mezzo extracellulare, che posseggono attività inibitoria verso batteri Gram positivi, in particolare ceppi strettamente correlati al microrganismo produttore. Poiché il loro spettro inibitorio comprende patogeni degli alimenti, nonché microrganismi responsabili del *food spoilage*, esse hanno il potenziale di additivi naturali degli alimenti nell'ottica di un approccio innovativo di conservazione biologica. Tipici bersagli delle batteriocine sono infatti microrganismi quali *Listeria monocytogenes* e *Staphilococcus aureus*, tipicamente associati a malattie di diversa natura, nonché batteri sporigeni come specie di *Bacillus* e *Clostridium*. Questi peptidi espletano la loro azione formando pori sulla membrana delle cellule bersaglio, quindi privandole del corretto potenziale transmembrana e gradiente di pH e causando la fuoriuscita di materiale cellulare.

Sulla base di caratteristiche prettamente strutturali, le batteriocine vengono tradizionalmente suddivise in tre classi. Alla classe I appartengono peptidi contententi amminoacidi inusuali come lantionina, β-metil-lantionina e deidroalanina; per questa loro caratteristica vengono anche chiamati lantibiotici. Il principale esempio di lantibiotico è sicuramente la nisina.

Alla classe II appartengono piccoli peptidi (PM<10 kDa), stabili al calore e privi di

lantionina e altri amminoacidi atipici. Questa classe è tradizionalmente suddivisa in tre sottogruppi. Alla classe IIa appartengono molte batteriocine con spiccata attività anti-*Listeria*, quindi di grande interesse applicativo.
La classe III, infine, è costituita da prodotti, ancora poco caratterizzati, ad alto PM (>30 kDa), labili al calore e di minor interesse applicativo.
L'impiego di batteriocine come biopreservanti, soprattutto nisina e peptidi di classe IIa, è stato ampiamente valutato, e si sta dimostrando una strategia efficace nel controllo di microrganismi patogeni. I principali approcci di conservazione biologica che sfruttano batteriocine sono:

1) inoculo dell'alimento con un batterio lattico (coltura protettiva) produttore di batteriocina (produzione *in situ*);
2) aggiunta diretta all'alimento di una batteriocina purificata o semi-purificata;
3) utilizzo di un prodotto precedentemente fermentato con un ceppo produttore di batteriocina.

La letteratura brevettuale è ricca di esempi che sfruttano questi metodi al fine di controllare la contaminazione dell'alimento da microrganismi patogeni e responsabili del deterioramento. I sistemi alimentari presi in considerazione spaziano da prodotti caseari (es. yogurt, latte e formaggi) a carni (es. salsicce e salami) a prodotti ittici (es. salmone) e vegetali (es. olive). Nonostante l'alto numero di lavori e brevetti prodotti, fino ad ora solo la nisina ha trovato reale applicazione ed è effettivamente prodotta attraverso un processo industriale e commercializzata. Questo è un peptide di 34 amminoacidi prodotto da ceppi di *Lactococcus lactis* ed è il rappresentante meglio studiato dei lantibiotici. La nisina normalmente ha attività inibitoria solo verso batteri Gram positivi, tra cui svariati batteri lattici, patogeni come *Listeria*, *Staphylococcus*, e *Mycobacterium*, e batteri sporigeni come *Bacillus* and *Clostridium*. Attualmente il suo utilizzo come biopreservante degli alimenti è accettato in circa 50 nazioni.
La carenza di altre effettive applicazioni commerciali di batteriocine è dovuta a problemi insiti nella tipologia di approccio considerato; in particolare le metodiche basate sull'utilizzo di colture protettive, sebbene più economiche rispetto all'utilizzo diretto di batteriocine come biopreservanti, sono limitate da un numero di fattori. In primo luogo, lo specifico ambiente dell'alimento (es. pH, temperatura, nutrienti) può non essere idoneo alla produzione della batteriocina. Inoltre, fattori limitanti possono essere la perdita spontanea della capacità di produrre il peptide e/o l'incapacità di secernere sufficienti quantità di prodotto tali da inibire i microrganismi indesiderati. Altre limiti sono la possibile contaminazione da fagi della coltura produttrice e l'antagonismo esercitato da altri batteri presenti nell'alimento, con conseguente inibizione della crescita del produttore e quindi della produzione di batteriocina. Vanno infine presi in considerazione eventuali effetti dannosi della coltura protettiva sulle qualità dell'alimento e sulla salute del consumatore (es. dovuti a produzione di tossine o ammine biogeniche).
Per via di questi limiti, gli approcci preferiti e più studiati sono quelli basati sull'aggiunta diretta di preparazioni purificate o semi-purificate della batteriocina di interesse. In questo caso la principale limitazione è dovuta alla mancanza di processi fermentativi idonei su larga scala necessari all'ottenimento di sufficienti quantità di prodotto. Inoltre, affinchè l'uso commerciale diventi effettivo, una strategia economicamente vantaggiosa per la purificazione della batteriocina dal brodo di fermentazione necessita di essere implementata ed ottimizzata.
Questo progetto di dottorato è nato proprio nella prospettiva di sviluppare processi

idonei alla produzione e purificazione su larga scala di batteriocine di interesse applicativo. In particolare tre batteri lattici, uno appartenente al genere *Enterococcus* e due al genere *Lactobacillus*, sono stati sottoposti a test di attività finalizzati a valutare la loro capacità di produrre batteriocine di interesse applicativo. Uno di essi, *Enterococcus casseliflavus* CGA1, si è mostrato capace di produrre un peptide con forte attività verso *Listeria monocytogenes*, candidandosi come microrganismo produttore di una batteriocina con potenziale applicativo quale biopreservante delle carni. Questo microrganismo, fornito da un'industria attiva nel settore alimentare, è stato infatti isolato da salami, dove *L. monocytogenes* comunemente espleta la sua attività patogenica.
Gli enterococchi sono batteri lattici isolati dal tratto intestinale dell'uomo e di una varietà di animali, nonché da diversi alimenti, soprattutto formaggi dell'area mediterranea e carni, quali salsicce fermentate e salami. E' interessante notare come molti ceppi enterococcali producano batteriocine, denominate enterocine, generalmente appartenenti alla classe II. Esse sono spesso attive verso altri enterococchi, ceppi di *L. monocytogenes* e specie di *Clostridium*. L'attività anti-*Listeria* può essere spiegata considerando la stretta parentela filogenetica esistente tra i due generi. Sulla base di questa forte attività, metodiche di conservazione che utilizzano enterococchi o enterocine sono molto promettenti. In quest'ottica, un processo di produzione fermentativa e purificazione dell'enterocina da *E. casseliflavus* CGA1 è stato sviluppato, ottimizzato e sottoposto a studi di *scale-up*.
La prima parte del lavoro è consistita nell'ottimizzazione della composizione di un terreno di coltura idoneo alla crescita del microrganismo e alla produzione di enterocina. E' infatti noto che la produzione di batteriocina è fortemente influenzata dalle condizioni di cultura, come pH, temperatura e composizione del mezzo. Solitamente condizioni che favoriscono la produzione di biomassa migliorano anche la produzione di batteriocina; pertanto, elevate rese di batteriocina sono state osservate in terreni ricchi, come MRS o MRS addizionato con ulteriori fonti complesse, come estratto di lievito, di malto o di carne. I limiti di questi terreni sono l'elevato costo e la composizione ricca tale da poter causare problemi di contaminazione nelle successive fasi di purificazione del prodotto; questi fattori ne rendono problematico l'utilizzo su scala industriale. Pertanto, fin dall'inizio ci si è orientati su un terreno a composizione semidefinita contenente una sola fonte complessa, quella di azoto fornito sotto forma di amminoacidi e peptidi. Esperimenti in beuta sono stati quindi effettuati allo scopo di individuare, tra una rosa di diversi peptoni industriali a basso costo, quello ottimale in termini di produzione di batteriocina. I risultati migliori sono stati ottenuti con i due peptoni di soia esaminati (indicati dalle sigle A2SC e E110): entrambi hanno permesso di ottenere, dopo circa 8 h di crescita, un massimo di attività di batteriocina di 80 BU (*Bacteriocin Units*)$\cdot mL^{-1}$. In termini di produzione specifica di batteriocina, che rappresenta la concentrazione di batteriocina prodotta per unità di biomassa (o densità ottica a 600 nm), il miglior dato è stato osservato in presenza di peptone di soia A2SC (20.5 $BU \cdot mL^{-1} \cdot OD^{-1}$). Come ci si poteva aspettare, un'alta produzione specifica (22.7 $BU \cdot mL^{-1} \cdot OD^{-1}$) è stata registrata anche quando il microrganismo è stato cresciuto in MRS.
Allo scopo di ridurre i costi del terreno, sono stati effettuati ulteriori esperimenti in beuta in cui il microrganismo è stato cresciuto in terreno semidefinito contenente diverse concentrazioni (da 20 a 5 $g \cdot L^{-1}$) di glucosio (fonte di carbonio e di energia) e di peptone di soia A2SC, i due principali componenti del mezzo. Una riduzione della produzione di biomassa del 30 % rispetto alle condizioni iniziali (20 $g \cdot L^{-1}$ per entrambi i nutrienti) è

stata osservata nei terreni contenenti 5 $g \cdot L^{-1}$ di peptone di soia A2SC, indipendentemente dalla concentrazione di glucosio (20, 10 o 5 $g \cdot L^{-1}$). Naturalmente, minore è risultata anche l'attività di enterocina, che da 80 è scesa a 60 $BU \cdot mL^{-1}$. Un dato invece interessante è che la produzione specifica di peptide aumenta nei terreni più poveri: un valore di 22.9 $BU \cdot mL^{-1} \cdot OD^{-1}$ è stato registrato nel terreno contenente un quarto delle concentrazioni iniziali dei due componenti. Essendosi quindi rivelato idoneo alla produzione ed economicamente vantaggioso, questo terreno è stato scelto e utilizzato per la crescita *E. casseliflavus* CGA1 nei successivi esperimenti di fermentazione.

Questi ultimi sono stati inizialmente effettuati in un fermentatore da 2 L esercito in modalità batch. E' stato possibile caratterizzare la curva di crescita del ceppo e di compararla alle cinetiche di consumo di glucosio e produzione di acido lattico. In tal modo parametri chiave, quali velocità di crescita specifica (μ) e rese di biomassa e di acido lattico su glucosio (Y_{xs} e Y_{ps}) sono stati determinati. Il valore ottenuto di Y_{ps} (0.89 $g \cdot g^{-1}$) mostra come il microrganismo intraprenda un metabolismo fermentativo di tipo omolattico quando cresciuto su glucosio. La cinetica di produzione di enterocina è stata anche caratterizzata: la produzione comincia appena la coltura entra in fase esponenziale fino a raggiungere un massimo di 80 $BU \cdot mL^{-1}$ a 6 h di crescita (picco dell'esponenziale), e rimanere costante durante la fase stazionaria; un decremento dell'attività si osserva in tarda fase stazionaria (24 h). Questo andamento riflette il carattere di tipico metabolita primario del prodotto, come del resto osservato per molte altre batteriocine. Anche la diminuzione di attività in fase stazionaria è riportata in letteratura e viene spiegata come conseguenza di inattivazione proteolitica e adsorbimento del peptide alla superfiicie cellulare del produttore, in maniera dipendente dal pH. Questo fenomeno è infatti prevalente a pH neutri (6-7), che sono anche i pH tipici di crescita dei batteri lattici, ed è minimo a pH acidi (2-3).

Essendo dunque la batteriocina un metabolita correlato alla crescita, maggiore è la produzione di biomassa, maggiore sarà l'attività di peptide. Pertanto, il passo successivo è consistito nel ricorso a strategie fed-batch di fermentazione per ottenere alte densità cellulari del microrganismo. Due differenti strategie di alimentazione sono state implementate. Nel primo caso, impulsi di terreno concentrato a tempi predefiniti e tali da restituire la concentrazione iniziale di ciascun componente hanno permesso di migliorare la produzione di biomassa e di enterocina (fino a 160 $BU \cdot mL^{-1}$ nell'esperimento con tre impulsi), ma la produzione specifica è risultata minore rispetto a quella osservata in batch. La seconda strategia è consistita nello sviluppo di un profilo continuo di alimentazione di substrato limitante (glucosio) all'interno del bioreattore, a partire dalla fine della fase batch. Questo profilo è stato determinato dai valori di velocità di crescita, resa di biomassa su substrato e consumo di glucosio ottenuti in batch. Con questa strategia una concentrazione finale (30 h) di biomassa di 11.6 $g \cdot L^{-1}$ è stata ottenuta, corrispondente ad un miglioramento di 8.5 volte di quella ottenuta in batch. Soprattutto, anche la produzione di enterocina, pari a 640 $BU \cdot mL^{-1}$, è stata sensibilmente migliorata (8 volte), così come la produzione specifica (30.4 $BU \cdot mL^{-1} \cdot OD^{-1}$) rispetto al batch. Sia in termini di biomassa che produzione di batteriocina, i dati ottenuti sulla piccola scala (2 L) sono stati riprodotti mediante una strategia di *scale-up* lineare anche su 15 e 100 L, a dimostrazione della validità e robustezza del processo anche su scale superiori.

Un processo biotecnologico economicamente attuabile richiede anche una idonea

strategia di purificazione del prodotto dal brodo di fermentazione. Le batteriocine vengono generalmente purificate mediante passaggi successivi di centrifugazione per allontanare le cellule, precipitazione in solfato d'ammonio per concentrare il peptide e uno o più passaggi cromatografici (soprattutto scambio ionico e fase inversa) per purificarlo ad omogeneità. Queste metodiche vanno bene per piccoli volumi (≤ 1 L), ma non sono proponibili per i grandi volumi di una produzione industriale. Metodi basati sull'utilizzo di solventi organici sono stati in questo caso messi a punto. Essi sono però molto costosi; inoltre, limiti legati al loro impatto ambientale e alla sicurezza dell'impianto vanno tenuti in considerazione. Pertanto, al fine di evitare il loro utilizzo, è stato sviluppato un processo innovativo di purificazione basato sulla tecnologia della filtrazione tangenziale. Il processo prevede due passaggi di ultrafiltrazione ed è costituito dalle seguenti operazioni: centrifugazione per allontanare le cellule; purificazione a 30 kDa per ritenere impurezze ad alto peso; precipitazione in solfato d'ammonio per concentrare il prodotto e ulteriore concentrazione e diafiltrazione a 3 kDa per l'allontanamento di sali ed altri metaboliti a basso peso. Questa procedura è stata comparata con processi di purificazione basati sull'utilizzo di solventi organici (butanolo/acetone e cloroformio/metanolo). Una resa di peptide del 75 % è stata ottenuta nella procedura con membrane, superiore a quelle dei due trattamenti con solventi organici (25 e 50 %, rispettivamente). Anche la purezza da altri contaminanti proteici è stata migliore con fattore di purificazione maggiore di 57, corrispondente ad un'attività specifica di circa 8700 unità di batterocina per mg di proteine. La validità delle procedura è stata valutata anche su un volume superiore (15 L). Utilizzando i medesimi parametri di processo (pressione transmembrana, flusso tangenziale, configurazione delle membrane) sono state ottenute simili prestazioni nei processi di membrana.

Il prodotto purificato è stato utilizzato per caratterizzare lo spettro di attività antimicrobica di *E. casseliflavus* CGA1 utilizzando come ceppi test sia batteri *starter* che patogeni. Oltre che verso *L. monocytogenes*, l'enterocina si è rivelata attiva anche verso colture *starter* di prodotti caseari, quali *Lactobacillus bulgaricus* DSM 21081 e *Lactobacillus casei* DSM 20011. Nessuna attività è stata invece osservata verso *Lactobacillus plantarum* DSM 12028, tipico *starter* delle carni isolato dai salami. Ovviamente l'assenza di attività verso microrganismi benefici dell'alimento è critica per una effettiva applicazione del prodotto come biopreservante. I dati ottenuti sembrano individuare nell'enterocina prodotta da *E. casseliflavus* CGA1 un promettente candidato come additivo naturale delle carni. Ulteriori prove devono essere effettuate per saggiare l'effettiva abilità del peptide di inibire microrganismi indesiderati nell'alimento.

Lo stesso spettro di attività è stato utilizzato per valutare le potenzialità inibitorie di due lattobacilli, *Lactobacillus plantarum* DSM 12028 e *Lactobacillus crispatus* L1. Il primo è uno *starter* delle carni, il secondo è un isolato vaginale con potenzialità probiotiche. Entrambi i microrganismi si sono rivelati capaci di inibire patogeni come *Staphilococcus aureus* e ceppi di *Escherichia coli*, in questo caso grazie all'azione combinata con EDTA, che rende accessibile alle batteriocine la membrana cellulare di batteri Gram negativi. Vista l'attività verso batteri patogeni, la produzione di batteriocine da questi due microrganismi è stata studiata più approfonditamente. Sono stati quindi effettuati esperimenti in beuta finalizzati a valutare l'effetto che concentrazioni diverse (1, 5 e 10 $g \cdot L^{-1}$) di bactocasitone, fonte complessa di azoto comunemente utilizzata per coltivare batteri lattici, hanno sulla produzione di batteriocina. Nel caso di *L. plantarum* DSM 12028, non vi è effetto tra 10 e 5 $g \cdot L^{-1}$. La produzione osservata di batteriocina è 40

$BU \cdot mL^{-1}$ in entrambi i terreni. L'effetto della concentrazione si è fatto sentire invece per *L. crispatus* L1: l'attività è dimezzata al diminuire della concentrazione di bactocasitone da 10 a 5 $g \cdot L^{-1}$. Nessuna produzione di batteriocina è stata invece osservata a concentrazione 1 $g \cdot L^{-1}$ di nutriente complesso, indicando come una alta quantità di fonte d'azoto sia essenziale per osservare una produzione basale di batteriocina.
Per entrambi i ceppi, sono stati inoltre effettuate crescite in fermentatore in modalità batch. L'ambiente di reazione più controllato ha permesso di migliorare la produzione di peptide. In particolare, nel caso di *L. plantarum* DSM 12028 è stato osservato un miglioramento di tre volte dell'attività, con una produzione specifica di 14.4 $BU \cdot mL^{-1} \cdot OD^{-1}$, superiore a quella ottenuta in beuta. Per *L. crispatus* L1, la produzione specifica misurata in fermentatore (circa 13 $BU \cdot mL^{-1} \cdot OD^{-1}$) è stata inferiore a quella ottenuta in beuta (15.3 $BU \cdot mL^{-1} \cdot OD^{-1}$), nonostante il miglioramento di due volte dell'attività.
Anche in questo caso il ricorso a strategie fermentative ad alta densità cellulare permetterà di migliorare ulteriormente la produzione di queste batteriocine di interesse applicativo.

CHAPTER I

1. INTRODUCTION

1.1 Food safety and preservation

Since the age of Louis Pasteur and Robert Koch, there has been scientific recognition of an essential need to control detrimental microorganisms in our environment. The discovery of penicillin by Alexander Fleming in 1929 opened the door for use of therapeutic antibiotics by the medical and veterinary communities to fight specific disease-causing organisms. Although therapeutic antibiotics are prohibited for use in foods, the utilization of antagonistic additives with preservative or antimicrobial properties has become a trademark approach (and a modern challenge) in food safety and preservation.

By definition, food preservation involves action taken to maintain foods with the desired properties or nature as long as possible (Raman S., 1999). It lies at the heart of food science and technology, and it is a main purpose of food processing.

Looking back at recent progress in food biotechnology, it is a fact that the problem of food security still remains to be solved. In spite of the introduction of modern technologies and safety concepts (e.g. HACCP), the reported number of food-borne illnesses and intoxications is still increasing. Estimates from the Centers for Disease Control and Prevention in the United States suggest that there are 76 million cases of food-borne illness the US each year, which results in about 5000 deaths (Cleveland et al., 2001). The US cost of food-borne illness associated with *Campylobacter jejuni*, *Clostridium perfringens*, *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus* and *Toxoplasma gondii* is between 6.5 and 34.9 $ billion (Buzby and Roberts, 1997). Recent outbreaks of emerging pathogens such as *L. monocytogenes* have prompted the food industry to question the adequacy of current methods of food preservation.

Approaches of food preservation were initially based on the utilization of chemically synthetized additives. On the other hand, consumers have been consistently concerned about possible adverse health effects from the presence of chemical additives in their foods. As a result, consumers are drawn to natural and “fresher” foods with no chemical preservatives added. This perception, coupled with the increasing demand for minimally processed foods with long shelfllife and convenience, has stimulated research interest in finding natural but effective preservatives. Recently, approaches of biological preservation of foods have become a well established weapon in the food preservation arsenal. The primary aim of these applications is of course the reduction of health risks without changing the qualities (e.g. sensory qualities) of the product.

1.2 Biological preservation of foods

Biological preservation implies a novel scientifically.based approach to improve the microbiological safety of foods. By definition, this concept refers to the use of antagonistic microorganisms (protective cultures) or their metabolic products (biopreservatives) to inhibit or destroy undesired microorganisms in foods to enhance food safety and prolong shelflife (Schillinger et al., 1996). A distinction is sometimes made between starter cultures and protective cultures; actually, it may be the same culture applied for different purposes under different conditions (Holzapfel et al., 1995). For a starter culture metabolic activity (e.g. acid production) has technological

importance whilst antimicrobial action may constitute a secondary effect; for a protective culture the functional objectives are inverse.
Lactic acid bacteria (LAB) have been involved in the fermentation and processing of a range of milk, meat, cereal and vegetable foods (McKay and Baldwin, 1990). In these foods, they are able to produce a large variety of compounds that contribute to their flavour, color, texture and consistency. Owing to their typical association with food fermentation and also their long tradition as food-grade bacteria, LAB also traditionally provide the major preservative effects in food processing which mankind has practiced for thousands of years.
LAB are a physiologically diverse group of organisms, which can be generally described as Gram-positve, nonsporing cocci or rods with lactic acid as the major product of carbohydrate fermentation. Traditionally, LAB comprise the four genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*. However, several new genera have been suggested for inclusion in the group of LAB due to a recent taxonomic revision (Axelsson S., 1998). The genus Streptococcus has been reorganized into *Enterococcus*, *Lactococcus*, *Streptococcus* and *Vagococcus*.
Modern approaches of biological preservation of foods that use LAB exploit the antimicrobial potential of protective cultures, food-grade enzymes and bacteriocins. As this research project is focused on the production and characterization of bacteriocins produced by some LAB strains, their use in food preservation will be discussed apart and more extensively.

1.2.1 Protective cultures

Protective cultures should in first place be considered as additional safety factor, with the potential of improving the microbiological safety of food. Their implementation should support good manufacturing practices (GMP), thereby reducing risks of growth and survival of pathogens and spoilage organisms. In addition under abuse conditions of temperature, handling, etc., their metabolic activities (e.g. acid or gas production) may serve as an indicator of microbial risk.
The LAB, generally considered as “food-grade” organisms, offer special promise for selection and implementation as protective cultures. Involved in numerous food fermentations known to man for millennia, it is assumed that most representatives of this group do not pose any health risk, and some are designated as GRAS (Generally Regarded As Safe) organisms. Reports on the involvement of LAB in human infections (Aguirre and Collins, 1993) indicate that some species may act as opportunistic pathogens in rare cases. However, there is no indication of a health risk of LAB associated with food fermentations. On the other hand, in a number of product groups, especially dairy products, the use of protective cultures may also contribute to the health benefits of a product. Such probiotic cultures are considered to provide substantial health benefits to man by means of stabilising or normalising the gastro-intestinal tract (Holzapfel et al., 1995). Some LAB strains are even associated with anticarcinogenic action and tumour control (Adachi S., 1992). These health traits may serve as important advantage for selection and development of protective cultures.

1.2.1.1 Mechanisms of antagonism of LAB protective cultures

Antagonism refers to the inhibition of other (e.g. undesired or pathogenic) microrganisms, caused by competition for nutrients and by the production of antimicrobial metabolites.
The production of one or more antimicrobially active metabolites is part of the complex mechanism by which a culture becomes established in the presence of other competing organisms. Understanding these mechanisms provides a valuable key towards biological approaches in food preservation. In Table 1 the antimicrobial properties of a number of metabolites from LAB are summarised. They include small metabolites, enzymes and bacteriocins. These last two categories will be discussed below in detail.
Depending on the product and processing, one or more of these metabolites may constitute a basis for the selection of a protective culture.

Organic acids. *Lactic acid*, the primary fermentation product of LAB, may reduce the pH to a level where pathogenic (e.g. salmonellae and *Listeria* spp.) and toxinogenic bacteria (*Staphylococcus aureus*, *Bacillus cereus*, *Clostridium botulinum*) will either be inhibited or destroyed. Moreover, the undissociated form will diffuse into the bacterial cell, thereby reducing the intracellular pH, thus slowing down metabolic activities. The rapid reduction of pH below 5.3 during raw sausage fermentation is sufficient to inhibit growth of salmonellae and *Staphylococcus aureus* (Schillinger and Lücke, 1988).
Due to its higher dissociation constant, *acetic acid* (pK_a 4.75) shows stronger inhibition than lactic acid (pK_a 3.1) at a given molar concentration and pH. Produced by heterofermentative LAB (*Leuconostoc* spp. and some lactobacilli) in equimolar amounts with lactic acid from hexoses sugars, acetic acid may constitute a vital factor for the establishment of the initial LAB population during several “spontaneous” fermentations (Buckenhüskes et al., 1990).
In a number of fermented dairy products, the *benzoic acid* concentration significantly exceeds the naturally expected value (Daeschel M.A., 1989). This constitutes an additional preservation factors in these dairy foods.

Hydrogen peroxide. Hydrogen peroxide is produced by a number of LAB in the presence of oxygen together with lactate, pyruvate and NADH (Kandler O., 1983). Undesired bacteria such as *Pseudomonas* spp. and *Staphylococcus aureus* are 2 to 10 times more sensitive to hydrogen peroxide than most LAB. In addition, this molecule may activate the lactoperoxidase system which is endogenous to fresh milk with the formation of hypothiocyanite and other antimicrobial products (Reiter and Härnulv, 1984).
As for other metabolic products, the tolerable amount of hydrogen peroxide is dependent on the product type, and may be detrimental to its qualities.

Low molecular weight metabolites. A number of primary metabolites of low molecular weight is known for their relatively potent antimicrobial activities.
Diacetyl is produced by some *Lactococcus*, *Leuconostoc* and *Pediococcus* spp. (e.g. during degradation of citric acid). Due to its intensive aroma, diacetyl has little direct potential for the preservation of food.
Carbon dioxide produced by heterofermenters from hexoses is directly toxic to aerobic putrefactive bacteria but may promote the growth of others.

Table 1
Antimicrobial compounds produced by lactic acid bacteria.

Product	Main target organisms
Organic acids	
➢ lactic acid	Putrefactive and Gram-negative bacteria, some fungi
➢ acetic acid	Putrefactive bacteria, clostridia, some yeasts and fungi
Hydrogen peroxide	Pathogens and spoilage organisms, especially in protein-rich foods
Enzymes	
➢ lactoperoxidase with H_2O_2	Pathogens and spoilage bacteria (dairy products)
➢ lysozyme	Undesired Gram-positive bacteria
Low molecular weight metabolites	
➢ reuterin	Wide spectrum of bacteria, moulds and yeasts
➢ diacetyl	Gram-negative bacteria
➢ fatty acids	Different bacteria
Bacteriocins	
➢ nisin	Some LAB and Gram-positive bacteria notably endospore-formers
➢ others	Gram-positive bacteria, inhibitory spectrum according to producer strain and bacteriocin type

Reuterin, or 3-hydroxypropionaldehyde, is produced from glycerol in *Lactobacillus reuteri*. It shows broad-spectrum antimicrobial activity, probably by inhibition of ribonucleotide reductase, and has been suggested for biopreservation of fish and meat, using *L. reuteri* (Daeschel M.A., 1989; Lindgren and Dobrogosz, 1990).

1.2.2 Food grade enzymes

The secretion of an antibacterial enzyme, such as lysozyme, may improve the activity spectrum of protective cultures, and may render such a strain more suitable for application under versatile conditions.
As for antimicrobial metabolites, a number of enzymes serve in nature to protect a biological system against invasion of certain microorganisms. Typical examples are lysozyme in egg albumen and lactoperoxidase in milk. Food systems which contain such antagonistic enzymes possess an intrinsic stability against microorganisms. On the other hand, purified antagonistic enzymes can be used as biopreservatives for foods which do not contain such antagonistic products, as for example the addition of lysozyme to cheese to prevent late blowing due to *Clostridium tyrobutyricum* (Bester and Lombard, 1990).
From a regulatory point of view, enzymes are considered as food additives and require special approval of “food grade” quality or GRAS status.

Lysozyme, one of the most important of them, can be found in milk and eggs. It is a muraminidase that hydrolyses β-1,4 linkages between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan, causing the lysis of bacterial cells. Gram-negative bacteria possess an outer membrane, which may protect the cell wall against the action of lysozyme. After disruption of the outer membrane by chelating membrane-stabilizing divalent cations with EDTA, also Gram-negative microorganisms become susceptible to the action of lysozyme.
Lysozyme is especially active against the outgrowth of clostridial spores but it shows activity against other pathogenic or toxinogenic bacteria such as *Bacillus* and *Listeria*. Investigations have especially concerned with control of the growth of *L. monocytogenes* (Carminati and Carini, 1989; Bester and Lombard, 1990). Lysozyme can control the growth of this microorganism in culture broth systems (Hughey and Johnson, 1987) and in real food systems like milk (Carminati and Carini, 1989) and cheese (Bester and Lombard, 1990), where it shows a remarkable stability.

Lactoperoxidase is an enzyme naturally occurring in milk. It represses the growth of *Staphylococcus aureus* and *Listeria monocytogenes* if activated in milk. It can be activated by thiocyanate ions and hydrogen peroxide. In the presence of both substrates lactoperoxidase oxidizes thiocyanate to hypothiocyanite. This is a strong oxidizing agent which in turn can oxidize essential sulfhydryl groups in bacterial proteins but is harmless to host cells (Kamau et al., 1990). Thiocyanate ions are present in milk and hydrogen peroxide can be produced by accompanying LAB.

Glucose oxidase oxidizes glucose to glucuronic acid in the presence of oxygen. In addition hydrogen peroxide is produced. This enzyme is already used in food industry to remove oxygen from various packaged or canned food products. It is in addition able to

repress the growth of certain Gram-positive as well as Gram-negative microorganisms. This inhibitory effect depends on the concentration of the enzyme in the medium and on glucose as substrate. Higher inhibitory activity can be observed at higher enzyme concentrations (Tiina and Sandholm, 1989). These experiments were performed in broth culture. On the other hand Jeong and co-workers (1992) demonstrated that glucose oxidase was not useful to inhibit the growth of *Pseudomonas* or *Salmonella* spp. on chicken breast skin. Glucose oxidase might be more active in liquid food systems.

Lactoferrin is an iron binding protein in milk, with activity towards *Bacillus subtilis*, *B. stearothermophilus* and *Escherichia coli*. These microorganisms are apparently inhibited due to the iron chelating capacity of the enzyme. Lactoferricin B is a proteolytic cleavage product of lactoferrin. It is a peptide of 25 amino acids and has much higher antagonistic activity than lactoferrin towards a lot of microorganisms, notably *Salmonella enteritidis*, *Campylobacter jejuni*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Clostridium perfrigens* (Bellamy et al., 1992).

1.3 Bacteriocins

As mentioned above, lactic acid bacteria are a source of antimicrobial peptides called bacteriocins, which have been examined for applications in microbial food safety. However, the bacteriocins were first characterized in Gram-negative bacteria. First discovered by Gratia in 1925, “principe V” was produced by one strain of *Escherichia coli* against another culture of *E. coli*. The term “colicine” was coined by Gratia and Fredericq in 1946; “bacteriocin” was subsequently used (1953) as a general term for highly specific antibacterial proteins. The term colicin now implies a bacteriocidal protein produced by varieties of *E. coli* and closely related Enterobacteriaceae (Konisky J., 1982).

Bacteriocins (as colicins) were originally defined as bacteriocidal proteins characterized by lethal biosynthesis, a very narrow range of activity, and adsorption to specific cell envelope receptors . Later, the recognized association of bacteriocin biosynthesis with plasmids was added to the description. The definition has since been modified to incorporate the properties of bacteriocins produced by Gram-positive bacteria (Tagg et al., 1976), notably LAB able to produce peptides with potential as effective natural food preservatives. Bacteriocins from Gram-positive bacteria commonly do not possess a specific receptor for adsorption, are most frequently of lower molecular weight than colicins, have a broader range of target bacteria with different modes of release and cell transport, and possess leader sequences cleaved during maturation (Riley M.A., 1998).

Currently, bacteriocins produced by LAB are defined as peptides or small proteins, ribosomally synthetized and extracellularly released, that display inhibitory activity towards Gram-positive bacteria, in particular closely related strains (Ennahar et al., 2000). Since their inhibitory spectrum often encompasses food pathogens and spoilers, they are natural additives for foods in the frame of an innovative food preservation (Jack et al., 1995). Although nisin is currently the only bacteriocin approved for use in many countries, a lot of bacteriocins produced by members of LAB have potential application in food products.

Bacteriocins are often confused in the literature with antibiotics (Hansen J.N., 1993). This would limit their use in food applications from a legal standpoint. So, it is critical to

make the distinction between bacteriocins and antibiotics. Their main differences are summarized in Table 2. Recognizing that bacteriocins are different from antibiotics, Hurst A. (1981), in his review, proposed the term “biological food preservatives” since bacteriocins, unlike antibiotics, are not used for medicinal purposes.

1.3.1 Classification of bacteriocins

Most of the bacteriocins from LAB are cationic, hydrophobic, or amphiphilic molecules composed of 20 to 60 amino acid residues (Nes and Holo, 2000). They are commonly classified into 3 groups that also include bacteriocins from other Gram-positive bacteria (Chen and Hoover, 2003). Examples of bacteriocins from these 3 classes are summarized in Table 3.

Class I. Class I is being further subdivided into class Ia and class Ib. In general, Class I peptides tipically have from 19 to more than 50 amino acids. Class I bacteriocins are characterized by their unusual amino acids, such as lanthionine, methyl-lanthionine, dehydrobutirine and dehydroalanine, so they are also called lantibiotics (from *lan*thionine-containing anti*biotic*). Class Ia bacteriocins, which include nisin, consist of cationic and hydrophobic peptides that form pores in target membranes and have a flexible structure compared to the more rigid class Ib. Class Ib bacteriocins, which are globular peptides, have no net charge or a net negative charge; antimicrobic activity is related to the inhibition of specific enzymes (Altena et al., 2000).

Class II. Small (<10 kDa), heat-stable, non-lanthionine-containing peptides are contained in class II. The largest group of bacteriocins in this classification system, these peptides are divided into 3 subgroups. Class IIa includes pediocin-like peptides having an N-terminal consensus sequence -Tyr-Gly-Asn-Gly-Val-Xaa-Cys. This subgroup has attracted much of the attention due to their anti-*Listeria* activity (Ennahar et al., 2000). Class IIb contains molecules requiring two different peptides for activity, and class IIc contains the remaining peptides of the class, including sec-dependent secreted bacteriocins (Nes et al., 2000).

Class III. The class III bacteriocins are not well characterized. This group includes large (>30 kDa), heat-labile proteins that are of lesser interest to food scientists.
A fourth class consisting of complex bacteriocins that require carbohydrate or lipid moieties for activity has also been suggested by Klaenhammer T.R. (1993); however, bacteriocins in this class have not been characterized adequately at the biochemical level to the extent that the definition of this class requires additional descriptive information (McAuliffe et al., 2001).

1.3.1.1 Properties and activity spectra of class I and class IIa Bacteriocins

Class I and class IIa bacteriocins are usually very stable at acidic pH. For example, Rodriguez and co-workers (2002) found that pediocin PA-1 was perfectly stable after 21 days of storage at 15 °C at pH 4 to 6; however, half of the activity was lost at pH 7.
In addition, bacteriocins from these two classes are heat stable at acidic pH. As pH increases, their heat stability decreases. Jack and co-workers (1996) found that heating

Table 2
Bacteriocins vs. antibiotics.

Characteristic	Bacteriocins	Antibiotics
Application	Food	Clinical
Synthesis	Ribosomal (primary metabolites)	Secondary metabolites
Activity	Narrow spectrum	Varying spectrum
Host cell immunity	Yes	No
Mechanism of target cell resistance or tolerance	Usually adaptation affecting cell membrane composition	Usually a genetically transferable determinant affecting different sites depending the mode of action
Interaction requirements	Sometimes docking molecules	Specific target
Mode of action	Mostly pore formation, but in few cases also cell wall byosinthesis	Cell membrane or intracellular targets
Toxicity/side effects	None known (rapidly digested by proteases in the human digestive tract)	Yes

Table 3
Examples of bacteriocins.

Group	Bacteriocins	Producer
Class Ia lantibiotics	Nisin, lacticin 481 Lactocin S Epidermin	*Lactococcus lactis* *Lactobacillus sake* *Staphylococcus epidermidis*
Class Ib lantibiotics	Mersacidin Cinnamycin Ancoverin	*Bacillus subtilis* *Streptomyces cinnamoneus* *Streptomyces ssp.*
Class IIa	Pediocin PA-1/AcH Sakacin A, sakacin P Mesentericin Y105 Enterocin A	*Pediococcus acidilactici* *Lactobacillus sake* *Leuconostoc mesenteroides* *Enterococcus faecium*
Class IIb	Lactococcin G, lactococcin M Plantaricin A, plantaricin S	*Lactococcus lactis* *Lactobacillus plantarum*
Class IIc	Acidocin Enterocin P, enterocin B	*Lactobacillus acidophilus* *Enterococcus faecium*
Class III	Helveticin J, helveticin V-1829	*Lactobacillus helveticus*

of piscicolin 126 for 120 min at pH 2 and 3 did not affect its bactericidal activity, while heating for 15 min at pH 4 or 5 reduced its activity by 50%.
In general, class I and class IIa bacteriocins are usually sensitive to proteolytic enzymes, such as trypsin, due to their proteinaceous nature.
Differences between two classes can be found in the activity spectra.
Most of the class I bacteriocins have a fairly broad inhibitory spectrum. They not only inhibit closely related bacteria, such as species from the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*, but also inhibit many less closely related Gram-positive bacteria, such as *L. monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridium botulinum* (Chen and Hoover, 2003). Interestingly, several bacteriocins in this class, such as nisin and thermophilin 13, also prevent outgrowth of spores of *B. cereus* and *C. botulinum*.
Compared to class I bacteriocins, most class IIa bacteriocins have comparatively narrow activity spectra and only inhibit closely related Gram-positive bacteria. In general, members of the genera *Enterococcus*, *Lactobacillus*, *Pediococcus* are sensitive to class IIa bacteriocins, and members of the genus *Lactococcus* are resistant. Some class IIa bacteriocins, such as pediocin PA-1, have fairly broad inhibitory spectra and can inhibit some less closely related gram-positive bacteria, such as *S. aureus* and vegetative cells of *Clostridium* spp. and *Bacillus* spp.
Interestingly, class IIa bacteriocins are generally active against Listeria. Eijsink and co-workers (1998) found that 9 strains of *Listeria* tested, including *L. monocytogenes*, *Listeria innocua*, and *Listeria ivanovii*, were very sensitive to four class IIa bacteriocins (namely pediocin PA-1, enterocin A, sakacin P, and curvacin A).
It might seem that bacteriocins with broader activity spectra would always be preferable for use in food preservation, but under certain circumstances the ones with narrower inhibitory spectra may prove more desirable. For example, sakacin P, which has limited activity against LAB but is nearly as effective as pediocin PA-1 against *Listeria*, might find application in LAB fermentation products that are prone to contamination by *L. monocytogenes* (Eijsink et al., 1998).

1.3.2 Genetics, biosynthesis and mode of action

1.3.2.1 Organization of gene clusters and biosynthetic pathway

The genes encoding bacteriocin production and immunity are usually organized in operon clusters. Bacteriocin gene clusters can be located on the chromosome, as in the case of mersacidin (Altena et al., 2000), on plasmids, as for sakacin A (Axelsson and Holck, 1995), or on transposons, as in the case of nisin (McAuliffe et al., 2001).
Typically, bacteriocin-producing LAB possess genes coding for the structural peptide, proteins that aid in processing to the active form, proteins deputated to the transport of the bacteriocin across the membrane, regulatory proteins, and proteins that confer immunity to the host producer.
Operons containing the genes for lantibiotic production are well studied, and homologous genes are found among the many of the sequenced lantibiotic operons, as reviewed by Siezen and co-workers (1996). Most characterized lantibiotic operons belong to class Ia.
The genetic regulation of many Class II bacteriocins has been also studied (Venema et

al., 1995; Diep et al., 1996) and shares many similarities with lantibiotics operons.
Though bacteriocins are ribosomally synthesized, the resulting product, a biologically inactive prepeptide carrying an N-terminal leader peptide that is attached to the C-terminal propeptide, must be modified before becoming active. Genes coding for the enzymes that facilitate the modifications are usually in close proximity to the structural gene.
For lantibiotics, extensive post-translational modifications include the formation of several unusual amino acids. It has been proposed that serine and threonine are modified to dehydroalanine and dehydrobutyrine, respectively, and that these amino acids serve as precursors to lanthionine and methyl-lanthionine, the formation of which occurs upon the addition of cysteine thiol groups (Kupke and Gotz, 1996).
The biosynthetic pathway of lantibiotics follows a general scheme: formation of prepeptide, modification reactions, proteolytic cleavage of the leader peptide, and the translocation of the modified prepeptide or mature propeptide across the cytoplasmic membrane, through a dedicated ABC-tranporter system (McAuliffe et al., 2001). Cleavage of the leader peptide may take place prior to, during, or after export from the cell.
Class II bacteriocins are synthetized as a prepeptide containing a conserved N-terminal leader and a characteristic double-glycine proteolytic processing site, with the exception of class IIc bacteriocins, which are produced with a typical N-terminal signal sequence of the sec-type and processed and secreted through the general secretory pathway. Unlike the lantibiotics, class II bacteriocins do not undergo extensive post-translational modification.
Following the formation of prepeptide, the prepeptide is processed to remove the leader peptide concomitant with export from the cell through a dedicated ABC-transporter and its accessory protein (Ennahar et al., 2000).

1.3.2.2 Mode of action

Bacteriocins, particularly lantibiotics, inhibit target cells by forming pores in the membrane, depleting the transmembrane potential and/or the pH gradient, resulting in the leakage of cellular materials (Okereke and Montville, 1992).
Bacteriocins are positively charged molecules with hydrophobic patches. Electrostatic interactions with negatively charged phosphate groups on target cell membranes are thought to contribute to the initial binding with the target membrane (Chen et al., 1997). The association of hydrophobic patches of bacteriocins with the hydrophobic membrane has also been modeled using computer simulation to predict the most favorable interaction (Lins et al., 1999). It is likely that the hydrophobic portion inserts into the membrane, forming pores
For other cationic non-lantibiotics peptides, the peptide concentration required to cause membrane depolarization does not always correspond with the minimal inhibitory concentration (MIC) and does not necessarily cause cell death (Friedrich et al., 2000). One may speculate that entry into the cell may be required to access targets such as DNA, RNA, enzymes and other sites to kill the target cell.
There is evidence that one Class II bacteriocin actually inhibits septum formation in susceptible bacteria (Martinez et al., 2000).
Because bacteriocins do not act equally against target species, researchers have

examined the affinity of bacteriocins to specific species and strains. The phospholipid composition of the target strains and environmental pH influence the MIC values (Chen et al., 1997). Differently from pore formation occurring indiscriminately, it appears that "docking molecules" on the target cell membrane facilitate the interaction with the bacteriocin, thereby increasing the effectiveness of the bacteriocin. This mechanism has been clearly demonstrated for nisin and mersacidin, which both use lipid II, a peptidoglycan precursor, as a docking molecule (Breukink et al., 1999). Mersacidin correspondingly inhibits peptidoglycan synthesis, whereas the primary mode of action of nisin is pore formation resulting in leakage of cellular materials. Figure 1 shows the nisin mode of action according to this model. Now, it is believed that in some cases cell wall biosynthesis may also be a target for nisin action.

1.3.2.3 Bacteriocin immunity

The immunity of the bacteriocin-synthesizing cell to its product is a phenomenon that distinguishes bacteriocins from antibiotics. Genes coding for so-called "immunity proteins" are in close genetic proximity to other bacteriocin structural and processing genes (Siegers and Entian, 1995). It is common for the structural bacteriocin gene and the immunity gene to be located on the same operon and often next to each other (Nes et al., 1996). In the class II bacteriocins, immunity protein is usually a basic protein between 50 and 150 amino acid residues long that is loosely associated with the membrane.
Despite the protection mechanism has not been unravelled yet, it is believed that immunity proteins bind to the membrane preventing interaction and/or pore formation caused by bacteriocins (Chen and Hoover, 2003).

1.3.3 Resistance mechanisms

The presence of an antibacterial substance in a given environment will eventually select for varieties of bacteria resistant to the antagonistic component. Cells already exhibit resistance to several antibiotics and the transfer of resistance between organisms has been documented.
Bacteriocin-resistant mutants also occur. Gravesen and co-workers (2002) examined the responses of a number of strains of *L. monocytogenes* to pediocin PA-1 and nisin, and found a wide range of resistances to the two bacteriocins occurring naturally. The influence of environmental stress (reduced pH, low temperature, and the presence of sodium chloride) appeared bacteriocin-specific; in particular, these stresses did not influence the frequency of resistance to pediocin PA-1, but the frequency of nisin resistance was significantly reduced. Interestingly, the stability of the phenotype of nisin resistance varied substantially, while resistance to the pediocin was stable with ongoing growth of *L. monocytogenes*.
The genes for synthesis of pediocin PA-1 were transferred into nisin-producing *L. lactis* FI5876 by Horn and co-workers (1999). As a result, both pediocin PA-1 and nisin A were synthesized concurrently in this strain of *Lactococcus*. Production levels of pediocin PA-1 were at the same levels as found in the original producer of the pediocin, *P. acidilactici* 347. Since pediocin PA-1 and nisin A are unrelated bacteriocins, use of *L. lactis* FI5876 as a starter culture in fermented dairy products should prevent the emergence of any

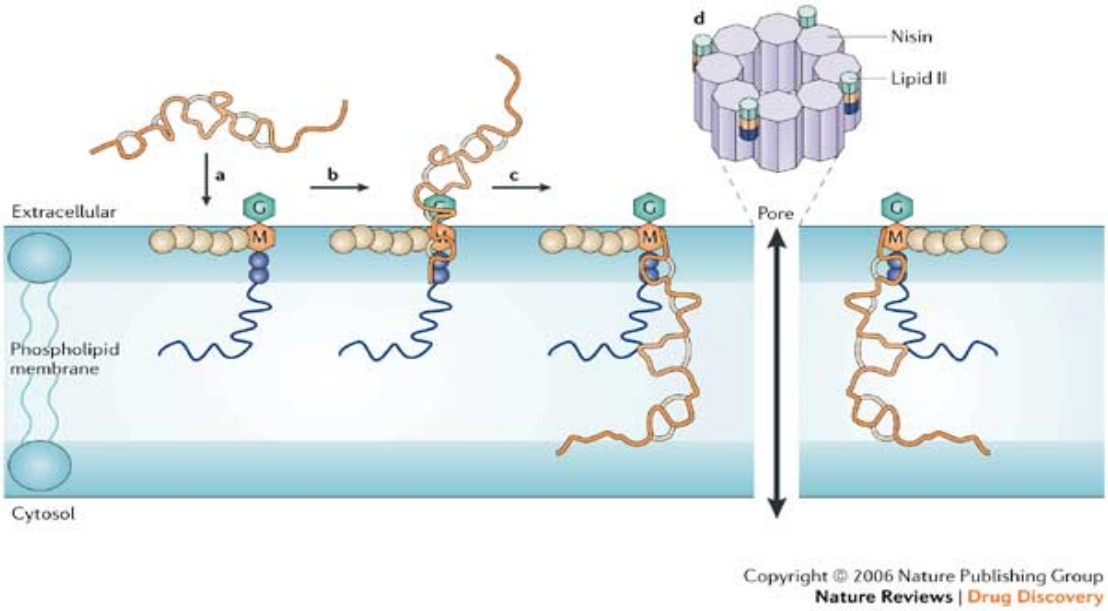

Figure 1
Proposed mode of action for nisin in according to “docking molecules” model (Breukink et al., 1999).

bacteriocin-resistant isolates of *L. monocytogenes* because the frequency for emergence of a strain resistant to both peptides should be low.
Although bacteriocins are not antibiotics, another question regards the resistance to antibiotics caused by exposure to bacteriocins. Since antibiotics and nisin have different modes of action, it has been shown that exposure to nisin has no effect on the frequency of resistance of *L. monocytogenes* to ampicillin and chloramphenicol (Tchikindas et al., 2000). In another study, following a different approach several multi-drug resistant bacteria were subjected to up to 400 IU/ml nisin, and these organisms remained sensitive to nisin (Severina et al., 1998).
Though bacteria exhibiting nisin resistance do not show cross-resistance with antibiotics, it is still important to understand the mechanism of resistance so that it can be avoided. Antibiotic resistance is usually associated with a genetic determinant, facilitating the transfer of resistance between cells. Unlike most antibiotic resistance, bacteriocin resistance results from a physiological change in the target cell membrane. For *L. monocytogenes*, a more rigid membrane, usually having a lower C15:C17 ratio results in increased tolerance to nisin (Crandall and Montville, 1998). Ming and Daeschel (1993) also found that nisin resistant *L. monocytogenes* have reduced amounts of phosphatidylglycerol, diphosphatidylglycerol and bisphosphatidylglycerol phosphate. Though most research show that a change in cell membrane composition accounts for resistance, some mutants produce an enzyme, nisinase, which degrades nisin (Jarvis B., 1967).

1.3.4 Determination of bacteriocin activity

Among the many methods described to assay antimicrobial activity, the methods reported below have been frequently used to determinate bacteriocin activity (Parente et al., 1995).

Agar diffusion assay. The agar diffusion method has long been used for testing antimicrobial activity, and it was first used by Fleming in 1924 (Piddock L.J., 1990). It includes agar well diffusion assay and disc assay. In this test, the bacteriocin solution is delivered to an agar plate (inoculated with a test microrganism) in a well or on a paper disc. The compound diffuses into agar resulting in a concentration gradient that is inversely proportional to the distance from the disc or well. The size of the inhibition zone around the disc or well is a measure of the degree of inhibition. The results of the test are generally qualitative. The method requires that the indicator organisms must grow rapidly, uniformly, and aerobically.
Several modified procedures based on the agar diffusion method have also been used for testing antimicrobial activity of LAB. These procedures include the agar spot test, deferred antagonism assay, and “spot on lawn” assay.

Critical dilution assay. Dilution methods are used as quantitative methods (Parente et al. 1995). The results are expressed as bacteriocin activity per mL (BU/mL), which is the lowest concentration of bacteriocin that prevents growth of a microorganism after a specific incubation period. In this test, the bacteriocin solution is serially diluted and each single concentration added to a plate inoculated with test organisms and incubated. The critical dilution is defined as the lowest concentration at which an inhibition halo occurs.

Turbidimetric assay. A turbidimetric assay based on automated systems determines the effect of an antimicrobial compound on the growth or death kinetics of a microorganism. Since the bacterial growth is monitored by measuring the turbidity of the broth medium, the method demands the instrument to be highly sensitive.
Skyttä and Mattila-Sandholm (1991) described a quantitative method based on automated turbidimetry for assaying antimicrobial activity, which was expressed as area reduction percentage values measured under the growth curve. The method has been used to test the antimicrobial activity of bacteriocins produced by *Lactobacillus plantarum* (Niku-Paavola et al., 1999).

1.4 Food applications of bacteriocins

Three approaches are commonly used in the application of bacteriocins for biological preservation of food (Chen and Hoover, 2003):

4) Inoculation of food with LAB (protective culture) that produce bacteriocin (production *in situ*). In this case, the ability of the LAB to grow and produce bacteriocin in the products is crucial for its successful use.
5) Addition of purified or semi-purified bacteriocins as food preservatives.
6) Use of a product previously fermented with a bacteriocin-producing strain as an ingredient in food processing.

Table 4 reports patented applications of these approaches. Although the number and variety of these applications is largely increasing, nisin remains the only bacteriocin whose application is approved for effective use in most countries. The use of pediocin PA-1 for food biopreservation has been commercially exploited and is covered by several U.S. and European patents. Fermentate containing pediocin PA-1, Alta^{TM}, is commercially available and used as a food preservative to increase shelflife and inhibit the growth of bacteria, especially *L. monocytogenes* in ready-to-eat meats (Rodriguez et al., 2002). Lacticin 3147, which is active over a wider pH range than nisin, is expected to find applications in non-acid foods (Ross et al., 1999).

1.4.1 Biopreservation of meat products

Listeria monocytogenes is a Gram-positive, non-sporeforming facultatively anaerobic rod widely distributed in the natural environment. It can grow over a pH range of 4.1 to 9.6 and a temperature range of 0 to 45 °C. The ubiquitous nature of *L. monocytogenes*, its hardness and ability to grow at refrigeration temperatures and anaerobic conditions make it a threat to the safety of foods. It is regarded as a major food safety problem because it can cause serious illnesses and death (Ryser and Marth, 1999). Many studies have been carried out to control *L. monocytogenes* in meat products since it is common within slaughterhouse and meat-packing environments and has been isolated from raw meat, and cooked and ready-to-eat meat products (Ryser and Marth, 1999).
Pawar and co-workers (2000) demonstrated that nisin, alone at concentrations of 400 and 800 IU·g^{-1}, significantly inhibited the growth of *L. monocytogenes* in minced raw buffalo meat. Addition of 2% sodium chloride in combination with nisin was found to increase the efficacy of inhibition.
Murray and Richard (1997) evaluated the antilisterial activity of nisin A and pediocin AcH in decontamination of artificially contaminated pieces of raw pork. Nisin A was

Table 4
Patented food applications of bacteriocins.

Authors	US Patent	Patent title	Description
Vandenbergh et al.	5,817,362 (06.10.98)	Method for inhibiting bacteria using a novel lactococcal bacteriocin	A method for inhibiting Gram-positive bacteria in foods by usng a novel bacteriocin produced by *L. lactis* NRRL-B-18535
Blackburn et al.	5,753,614 (19.05.98)	Nisin compositions for use as enhanced, broad range bactericides	Combination of nisin, a chelating agent and a surfactant to inhibit both Gram-positive and Gram-negative organisms in meat, eggs, cheese and fish
Wilhoit	5,573,801 (12.11.96)	Surface treatment of foodstuffs with antimicrobial compositions	Use of *Streptococcus*-derived or *Pediococcus*-derived bacteriocins in combination with a chelating agent to protect food against *Listeria*
Vedamuthu	5,445,835 (29.08.95)	Method of producing a yogurt product containing bacteriocin PA-1	A yogurt product with increased shelf life containing a bacteriocin derived from a *P. acidilactici*
Boudreaux et al.	5,219,603 (15.06.93)	Composition for extending the shelf life of processed meats	Use of a bacteriocin from P. acidilactici and a propionate salt to inhibit bacterial growth and to extend shelf life of raw and processed meat
Hutkins et al.	5,186,962 (16.02.93)	Composition and method for inhibiting pathogens and spoilage organisms in foods	Use of bacteriocin-producing LAB to inhibit growth of food-born pathogens
Collison et al.	5,015,487 (14.05.91)	Use of lanthionines for control of post-processing contamination in processed meats	Inhibiting the contamination of processed meat products by pathogenic or spoilage microorganisms by treating the surface of the meat product with a lantibiotic
Vandenbergh et al.	4,929,445 (29.05.90)	Method for inhibiting *L. monocytogenes* using a bacteriocin	Inhibition of *L. monocytogenes* by a bacteriocin produced by *P. acidilactici*
Gonzalez	4,883,673 (28.11.89)	Method for inhibiting bacterial spoilage and resulting compositions	Inhibition of food spoilage microorganisms in salads and salad dressings by a bacteriocin from *P. acidilactici*
Matrozza et al.	3,790,994 (13.12.88)	Method for inhibiting psychrotrophic bacteria in cream or milk based products using a *Pediococcus*	Inhibition of bacterial growth in cottage cheese by a bacteriocin-producing *P. pentosaceus* cells

considerably more efficient than pediocin AcH, but after 2 days of storage, surviving bacteria in meat treated with each bacteriocin resumed growth at a rate similar to that of the control. Moreover, nisin was more stable than pediocin AcH. The loss of effectiveness, especially of pediocin AcH, was attributed to rapid degradation by meat proteases.
In addition to nisin and pediocin, other LAB bacteriocins have been examined to control the growth of *Listeria*. Examples include Lauková and co-workers (1999), who examined the effectiveness of enterocin CCM 4231 in controlling *L. monocytogenes* contamination in dry fermented Hornád salami. Addition of enterocin reduced the counts of *L. monocytogenes* by 1.7 orders of magnitude immediately after addition of the bacteriocin.
Also, Hugas and co-workers (1998) found that sakacin K, a bacteriocin produced by *Lactobacillus sake* CTC494, inhibited the growth of *Listeria innocua* in vacuum-packaged samples of poultry breasts and cooked pork, and in modified atmosphere-packaged samples of raw minced pork.
Degnan and co-workers (1992) demonstrated the possibility of using protective cultures of *Pediococcus acidilactici* (pediocin AcH producer) to control *L. monocytogenes* growth in vacuum packaged all-beef wieners.
The primary reasons that nitrite is commonly used in curing meats is to stabilize red meat color and inhibit food spoilage and poisoning organisms, such as *Clostridium botulinum*; however, nitrite can react with secondary amines in meats to form carcinogenic nitrosamines. This possible adverse health effect has prompted researchers to explore the potential of using bacteriocins as an alternative to nitrite. Rayman and co-workers (1981) reported that a combination of 3000 $IU \cdot g^{-1}$ of nisin and 40 ppm of nitrite almost completely inhibited outgrowth of *Clostridium sporogenes* spores in meat slurries at 37 °C for 56 days; reducing the pH was found to enhance nisin activity.

1.4.2 Biopreservation of diary products

L. monocytogenes has been the documented cause of a number of outbreaks associated with dairy products, such as pasteurized milk and cheese, and nisin has been shown effective against this organism in dairy products.
Ferreira and Lund (1996) found that following inoculation of a nisin-resistant strain into long-life cottage cheese at pH 4.6 to 4.7, the number of *L. monocytogenes* decreased approximately by one order of magnitude for 7 days. Addition of nisin (2000 $IU \cdot g^{-1}$) to the cottage cheese increased the rate of inactivation to approximately 3 orders of magnitude in 3 days.
Davies and co-workers (1997) determined the efficacy of nisin to control *L. monocytogenes* in ricotta-type cheeses over 70 day. Addition of nisin effectively inhibited the growth of *L. monocytogenes* for a period of 8 weeks or more (dependent oncheese type).
Zottola and co-workers (1994) used nisin-containing cheddar cheese that had been made with nisin-producing lactococci as an ingredient in pasteurized process cheese or cold pack cheese spreads. The shelflife of the nisin-containing pasteurized process cheese was significantly greater than that of the control cheese spreads. In cold pack cheese spreads, nisin significantly reduced the numbers of *L. monocytogenes*, *S. aureus* and spores of *C. sporogenes*.

Another problem in cheese production is the *Clostridium*-associated butyric acid fermentation. Nisin is commonly added to pasteurized processed cheese spreads to prevent the outgrowth of clostridia spores, such as *Clostridium tyrobutyricum* (Schillinger et al., 1996).

1.4.3 Biopreservation of seafood products

The effectiveness of bacteriocins and protective cultures to control growth of *L. monocytogenes* in vacuum-packed coldsmoked salmon has been demonstrated by several researchers.
Katla and co-workers (2001) examined the inhibitory effect of sakacin P and/or *L. sake* cultures (sakacin P producer) against *L. monocytogenes* in cold-smoked salmon. Sakacin P had an initial inhibiting effect on growth of *L. monocytogenes* while cultures of *L. sake* had a bacteriostatic effect. When *L. sake* culture was added to salmon together with sakacin P, a bacteriocidal effect against *L. monocytogenes* was observed.
The inhibitory effect of nisin in combination with carbon dioxide and low temperature on the survival of *L. monocytogenes* in cold-smoked salmon has also been investigated (Nilsson et al., 2000). Addition of nisin to salmon inoculated with *L. monocytogenes* and stored at 5 °C delayed, but did not prevent growth of *L. monocytogenes* in vacuum-packs. Addition of nisin to CO_2-packed cold-smoked salmon resulted in one to two oreders of magnitude reduction of *L. monocytogenes*.
In order to improve shelflife, brined shrimp are typically produced with the addition of sorbic and benzoic acids. Concerns about the use of these organic acids have led researchers to explore the potential of using bacteriocins for their preservation. The effectiveness of nisin Z, carnocin UI49, and a preparation of crude bavaricin A on shelflife extension of brined shrimp was evaluated by Einarsson and Lauzon (1995) demonstrating how the inhibition by bacteriocin multiple addition is comparable to organic acids effects.

1.4.4 Nisin

Nisin is 34–amino acid peptide produced by strains of *Lactococcus lactis* and is the best studied representative of the lantibiotics (Klaenhammer T.R., 1993). At least 6 different forms have been discovered and characterized (designated as A through E and Z), being nisin A the most active type. Nisin usually has no effect on Gram-negative bacteria, yeasts and moulds, although Gram-negative bacteria can be sensitized to nisin by permeabilization of the outer membrane layer as caused by heating, freezing, and chelating agents (Delves-Broughton et al., 1996). Normally only Gram-positive bacteria are affected, and these types include LAB, pathogens such as *Listeria*, *Staphylococcus*, and *Mycobacterium*, and the sporeforming bacteria, *Bacillus* and *Clostridium*.
In 1928, Rogers and Whittier first published on the inhibitory effect of nisin in which *Streptococcus lactis* (now *Lactococcus lactis* subsp. *lactis*) inhibited *Lactobacillus bulgaricus* in milk. The name nisin was coined by Mattick and Hirsch (1947) from “N inhibitory substance” since *L. lactis* was originally classified as Lancefield serological group N *Streptococcus*. Hirsch and co-workers (1951) first examined the potential of nisin as a food preservative. In 1957, Aplin and Barrett developed commercial preparations containing nisin for use in foods (Delves-Broughton et al., 1996). Finally,

international acceptance of nisin was given in 1969 by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives. Currently, It has been accepted as a food additive in approximately 50 countries.
The largest producer is still Aplin & Barrett Ltd (Dorset, England, a subsidiary of Cultor Food Science, Ardsley, New York, USA), which markets a standardized preparation of nisin, Nisaplin^{TM}, with the active ingredient 2.5% nisin A and the predominate ingredients NaCl and skimmed dry milk. In the current industrial process, pasteurized milk plus added yeast extract is treated with a protease and used as a substrate in batch fermentation at controlled pH and temperature. An extraction process, including a spray-drying step, follows and the resulting powder is standardized with NaCl. Further purification is expensive and not commercially valuable for food use. A standardized nisin preparation (Chrisin) is also marketed by Chr Hansen (Hùrsholm, Denmark).
Examples of international food products that legally can be amended with nisin are canned soups (Australia), ice for storing fresh fish (Bulgaria), clotted cream (UK) and milk shakes (Spain); however, the majority of approved product types are dairy products (especially cheeses) and canned goods (Guder et al., 2000).
In the U.S., use of nisin-producing starter cultures has never been regulated, as lactococci are considered GRAS. As inferred from the FDA Code of Federal Regulations (1988) regarding use of bacteriocins as food additives, when natural antimicrobials (that is, bacteriocins such as nisin) are used as food preservatives, the producing culture must be considered a GRAS microorganism (see nextly, “Regulatory considerations”).

1.4.5 Use of bacteriocins in hurdle technology

The major functional limitations for the application of bacteriocins in foods are their relatively narrow activity spectra and moderate antibacterial effects. To overcome these limitations, more and more researchers use the concept of hurdle technology to improve shelflife and enhance food safety (Chen and Hoover, 2003; Leistner L., 2000). Hurdle technology combines different preservation methods to inhibit microbial growth. For example, they are generally not active against Gram-negative bacteria. Indeed, it is well documented that Gram-negative bacteria become sensitive to bacteriocins if the permeability barrier properties of their outer membrane are impaired. For example, chelating agents, such as EDTA, can bind magnesium irons from the lipopolysaccharide layer and disrupt the outer membrane of Gram-negative bacteria, thus allowing nisin to gain access to the cytoplasmic membrane (Zhang and Mustapha, 1999).
It is well documented that nisin enhances thermal inactivation of bacteria, thus reducing the treatment time and resulting in better food qualities. For example, Boziaris and co-workers (1998) found that addition of nisin (500 to 2500 IU·ml^{-1}) in media, liquid whole egg, or egg white caused a reduction of required pasteurization time up to 35%. Budu-Amoako and co-workers (1999) found that nisin reduced the heat resistance of *L. monocytogenes* in lobster meat and significantly reduced the treatment time compared with thermal treatment alone. The reduced heat process resulted in significant reduction in drained weight loss that would allow considerable cost savings.
The synergistic effect between bacteriocins and other processing technologies on the inactivation of microorganisms has also been frequently reported in the literature. Schlyter and co-workers (1993) reported synergistic effects between sodium diacetate

and pediocin against *L. monocytogenes* in meat slurries.
Zapico and co-workers (1998) showed a synergistic effect of nisin and the lactoperoxidase system on inactivation of *L. monocytogenes* in skim milk. Addition of nisin and lactoperoxidase system resulted in counts of *L. monocytogenes* up to 5.6 orders of magnitude lower than the control milk after 24 h at 30 °C, while nisin alone had no effect on the counts. The use of combinations of various bacteriocins has also been shown to enhance antibacterial activity (Hanlin et al., 1993). When used in combination with nisin, leucocin F10 provides greater activity against *L. monocytogenes* (Parente et al., 1998).
There has been continued interest in the food industry in using nonthermal processing technologies, such as high hydrostatic pressure (HP) and pulsed electric field (PEF) in food preservation. It is frequently observed that bacteriocins, in combination with these processing techniques, enhance bacterial inactivation (Terebiznik et al., 2000). In addition, Gram-negative bacteria that are usually insensitive to LAB bacteriocins, such as *Escherichia coli* O157:H7 and *Salmonella typhimurium*, become sensitive following HP/PEF treatments that induce sublethal injury to bacterial cells (Kalchayanand et al., 1994).

1.4.6 Regulatory considerations

From a regulatory standpoint, it is critical to distinguish bacteriocins from antibiotics, since the presence of antibiotics in food is often prohibited. For example, in Denmark, bacteria used to produce food additives must not produce toxins or antibiotics (Cleveland et al., 2001).
The use of bacteriocin-producing starter cultures as ingredients may not require special consideration in the United States if the culture (microrganism) is considered GRAS, because of its history of safe use by food industries prior to the 1958 Food Additives Amendment (Muriana P., 1996).
If a purified bacteriocin is used as a food preservative, the substance might be self-affirmed as GRAS by the company according to the Code of Federal Regulations (U.S. Government Printing Office, 1990), but the Food and Drug Administration (FDA) may require justification of the affirmation.
With the formation of the European Union, food additives have been given “E” numbers. Nisin is listed as E234, and may also be labeled as “nisin preservative” or “natural preservative”.
In the United States, where antibiotics are prohibited in foods, nisin was confirmed Generally Recognized as Safe in 1988 (U.S. Food and Drug Administration). Several authors have outlined issues involved in the approval of new bacteriocins for food use (Post R., 1996) and the USDA publishes guidelines for the safety assessment of a new preservative (U.S. Food and Drug Administration, 1993). The Food Safety and Inspection Service (FSIS) of the USDA evaluates the safety and effectiveness of bacteriocins in commodity segments such as meat and poultry products. Thus depending on the type of product, the FDA or FSIS approve use of novel bacteriocins before any applications are permitted (Chikindas and Montville, 2002).
For approval to be granted, the bacteriocin must be chemically identified and characterized, and its use and efficacy must be shown. The manufacturing process must be described and assays used for quantification and standardization of the peptide must

be shown. Toxicological data and the fate of the molecule after ingestion are also needed.

1.5 Bacteriocins produced by *Enterococcus* spp.

The genus *Enterococcus* comprises up to date 26 different species, with *Enterococcus faecium* and *Enterococcus faecalis* being the two species mostly found (Klein G., 2003). They are traditionally considered part of LAB. Especially their ubiquitous occurrence and their habitats in the intestinal tract of mankind and a variety of farm animals as well as in different sorts of food and feed are typical for LAB.
In particular, enterococci frequently occur in some cheeses (Sarantinopoulos et al., 2002; Giraffa G., 2003), above all in those produced in Mediterranean countries, such as Feta and Mozzarella, and in meats (Hugas et al., 2003), such as dry fermented sausages. In these foods, they are considered as either a contamination from the environment or a natural starter culture, contributing to the processing and the development of the organoleptic properties of the product. Moreover, *Enterococcus* spp. are occasionally used as probiotics (Franz et al., 2003).
Interestingly, many strains of enterococci are known to produce bacteriocins. These are called enterocins or enterococcins and they generally belong to class II (Giraffa G., 1995). The enterocins are generally active against other enterococci as well as strains of *Listeria monocytogenes* and *Clostridium* spp., including *C. botulinum*, *C. perfrigens* and *C. tyrobutyricum*. The anti-*Listeria* activity may be explained by the fact that enterococci and listeriae are phylogenetically closely related (Devriese and Pot, 1995). Bacteriocin-producing *Enterococcus* strains with strong anti-*Listeria* activity have been isolated from dairy products (Giraffa G., 2003), fermented sausages (Hugas et al., 2003), fish (Ben Embarek et al., 1984), vegetables (Bennik et al., 1998) and fermented olives (Franz et al., 1996).
Examples of the best characterised enterocins are enterocin A, a class IIa, pediocin-like bacteriocin (Aymerich et al., 1996), and enterocin B (Casaus et al., 1997), a bacteriocin that is not pediocin-like, but it is similar to the class IIa bacteriocins because of its chemical characteristics, heat stability and anti-*Listeria* activity. Enterocin P belongs to class IIc because secretion occurs by the sec-pathway (Cintas et al., 1997). Enterocins L50A and L50B have intrinsic antimicrobial activity, but interestingly together they show synergistic activity. They are not post-translationally modified and do not require the presence of a leader or signal peptide for secretion (Cintas et al., 1998).
The application of bacteriocins produced by enterococci and of bacteriocin-producing *Enterococcus* starters or protective cultures is well documented in cheese and meat manufacture. Bacteriogenic *E. faecium* was tested for use in combination with a commercial starter culture for Taleggio cheese making (Giraffa G., 1995). Bacteriocin was produced during drainage in the cheese until the end of the ripening period, while growth of the commercial starters was not inhibited. Hugas and co-workers (2003) reported that the production of enterocin A and B by *E. faecium* CTC492 was very low in the typical conditions of sausage manufacturing. Despite this, the addition of these enterocins to the fermented sausages spiked with *Listeria monocytogenes* significantly diminished *Listeria* counts compared with the control. Other bacteriogenic strains of *E. faecium* (RZS C13 and CCM4231) and *E. faecalis* AS48 were assayed as anti-listerial protective cultures in real or model sausage fermentations (Callewaert et al., 2000). All

Enterococcus strains inhibited *Listeria* further than the positive control.
Based on these studies and due to their strong activity against *Listeria* spp., potential approaches of food preservation using enterocins-producing protective enterococci or purified enterocins as biopreservatives are very promising.

1.6 Productive processes of bacteriocins

Altough many food preservation applications exploiting the ability of producing bacteriocin by LAB use protective cultures (production *in situ*), these approaches are limited by a number of factors (Schillinger et al., 1996). First of all, environmental parameters necessary for food production (e.g. pH, temperature, nutrients) might be not suitable for bacteriocin synthesis and/or release. Moreover, the spontaneous loss of bacteriocin-producing ability and the inability to release sufficient quantities of the bacteriocin to suppress undesired bacteria in food environment are also key factors to be considered in the development of a protective culture. Other limits to this approach are eventual phage infection of the producing culture and the antagonism by the other bacteria present in food, thus inhibiting the microrganism growth and consequently the bacteriocin production. Potential deleterious effects of the protective cultures on the sensorial qualities and the consumer health (e.g. by toxins or biogenic amines production) are also drawbacks to be overcome.
Due to these limits, most studied and preferred applications recently imply the direct addition of semi-purified or (better) purified bacteriocin as food biopreservative.
In order to make the commercial use of these bacteriocin preparations effective, the development of an appropriate fermentative process is required to obtain high yield of product. In parallel, an economically feasible strategy for purification of the bacteriocin from the fermentation broth has to be studied and optimized.

1.6.1 Fermentative processes of bacteriocins

1.6.1.1 Kinetics of bacteriocin production

Generally, bacteriocin production shows primary metabolite kinetics, starting as soon as cell growth begins and ceasing at or immediately before the end of cell growth (Parente et al., 1997). This implies that the bacteriocin production is dependent on the total biomass formation. However, a high cell yield does not necessarily results in a high bacteriocin activity since the latter may be limited by a low specific bacteriocin production, i.e. a low bacteriocin production per gram of cells (Leroy and De Vuyst, 1999). Hence, a rather complex relationship between environmental conditions and bacteriocin production exists. An interesting example of this behaviour is purchased by Leroy and De Vuyst (2002). They demonstrated that enterocin production by *E. faecium* RZS C5 grown on MRS medium at pH 6 in batch modality is limited to the very early growth phase and is switched off at a well defined cell density. On the other hand, at constant pH values higher than 6.5, enterocin production is lower but maintained until the end of the exponential phase. This behaviour suggests as the enterocin production is strongly regulated.
After reaching a maximal bacteriocin activity during the exponential growth phase, a drastic decrease in bacteriocin activity often occurs as a consequence of proteolytic

inactivation, protein aggregation and pH-dependent adsorption of the bacteriocin molecules to the cell surface of the producer cells (Yang et al., 1992). Since adsorption of bacteriocins to cells is maximal at pH 5.5-6.5 and decreases at low pH, it is not surprising that no reduction of bacteriocin titre is sometimes observed in fermentations without pH control (Yang and Ray, 1994).

1.6.1.2 Factors affecting bacteriocin production in LAB

Any factors increasing cell growth should increase bacteriocin production, but the yield of bacteriocin per unit of biomass is strictly affected by several factors (Parente and Ricciardi, 1999), including the producing strain, media (carbohydrate and nitrogen sources, cations, etc.) and fermentation conditions (pH, temperature, stirring, aeration, feeding profile in fed-batch fermentations and dilution rate in continuous fermentations).

Microbial strain. A given bacteriocin can be produced by several strains or species (Jack et al. 1995). Yang and Ray (1994) found that nisin and leuconocin Lcm1 production varied among different strains, while pediocin AcH showed less variation. De Vuyst L. (1994) screened 21 nisin-producing and 6 non-producing strains of *L. lactis* for nisin production and immunity. Nisin titres varied from 1 to 1886 $IU \cdot ml^{-1}$ and did not correlate with the number of copies of the genes or with their transcription or translation levels. Differences among strains were attributed to expression level and activity of maturing enzymes and, to a lesser extent, to nisin immunity.

Media. Bacteriocin production is deeply affected by type and level of the carbon, nitrogen and phosphate sources, cations, surfactants and inhibitors (Parente and Ricciardi, 1999).
Since LAB are nutritionally fastidious microorganisms, growth and bacteriocin production is often increased in complex media (e.g. MRS). In a recent work, Todorov and Dicks (2006) evaluated the bacteriocin production by two strains (ST23LD and ST341LD) in different media and pH conditions. They observed that addition of tryptone, yeast extract and meat extract to a basal medium constituted by MRS have beneficial effects on the bacteriocin production. Comparison of bacteriocin production by some probiotic LAB in MRS and milk-based medium showed a greater production in the first one (Avonts et al., 2004). Nevertheless, the addition of yeast extraxt to the milk can enhance the bacteriocin production.

Effect of fermentation conditions. Since pH and temperature control improve the growth of LAB, it also results in improved bacteriocin production. However, the optimal pH for bacteriocin production is usually 5.5-6 (Parente and Ricciardi, 1999), often lower than the optimal pH for growth. On the other hand, growth at optimal temperature usually results in optimal bacteriocin production but temperature stress and growth at sub-optimal temperature may result in an increase of bacteriocin yield per biomass unit (Lejeune et al., 1998).
Agitation and aeration also affect bacteriocin production. Increases of these parameters often result in decrease of bacteriocin activity, perhaps because of chemical degradation and effects on gene expression (Parente and Ricciardi, 1999).
Researchers generally exploit batch fermentations to find best conditions (pH, T,

aeration and agitation) for bacteriocin production, also exploiting mathematical modeling. Leal-Sánchez and co-workers (2002) optimized conditions (glucose and NaCl concentrations, inoculum size, aeration and temperature) of batch fermentation of *Lactobacillus plantarum* LPCO10 using a statistical approach; under optimal conditions, a substantial improvement of product was achieved.
Batch fermentations were also used to optimize pH and temperature for bacteriocin production by *Lactobacillus curvatus* LTH 1174 and to validate a model that describes the influence of these parameters on microbial behaviour (Messens et al., 2003).
These studies demonstrate that batch fermentations are useful to find the cultural conditions that maximize bacteriocin production, but the overall product yield remains low. Differently from batch fermentations, where the bacteriocin production ceases at the end of growth phase, a way to improve bacteriocin production is the fed-batch fermentation technology, that allows to obtain high cell densities through the supply of fresh medium. Fed-batch fermentations to improve bacteriocin production were described for nisin, gallidermin and epidermin (Ungermann et al., 1991). In these works, an appropriate feeding strategy allows to enhance the bacteriocin production. Interestingly, Callewaert and De Vuyst (2000) found that production of amylovorin L471, a bacteriocin produced by *Lactobacillus amylovorus* DCE 471, is improved and stabilized in fed-batch fermentation if substrate addition is controlled by acidification rate of the culture. A comparison of the performance of different fermentation strategies (flask, batch and fed–batch with constant or exponential feeding) on growth and bacteriocin production by *L. curvatus* CWBI-B28 was done by Ghalfi and co-workers (2007); the best bacteriocin activity was obtained in a very rich medium fed into bioreactor following an exponential profile.
Occasionally, continuous cultures were also applied to study the effect of growth rate on bacteriocin production (Parente et al., 1997; Bárcena et al., 1998). A linear relationship is often observed between dilution rate (D) and bacteriocin production rate. Increase in D results in a decrease of biomass and bacteriocin concentration, a high substrate concentration in the effuent, and eventually wash-out of the culture. Therefore, continuous fermentation with cell recycle or with immobilized cells has been tested by some authors. Continuous fermentation coupled with cell recycle with *L. lactis* subsp. *lactis* IFO12007 led to an increase in nisin level and volumetric nisin productivity compared to batch fermentations (Taniguchi et al., 1994). Wan and co-workers (1995) compared nisin, brevicin 286 and pediocin PO2 production with free or Ca-alginate immobilized cells. In repeated batch fermentations, immobilized cells produced less bacteriocin than free cells, perhaps because of diffusional limitations of the substrate in alginate gels.
An interesting comparison of different reactor designs to improve bacteriocin productivity was performed by Bhugaloo-Vial and co-workers (1997). Three different reactors (a chemostat with free cells, a chemostat with cells immobilized in calcium-alginate beads, and a membrane bioreactor) were used, showing that immobilized cells presented the best performances in terms of bacteriocin productivity and concentration.

1.6.2 Downstream processes of bacteriocins

The use of bacteriocins as biopreservatives directly added to foods requires the development of a simple and rapid method by which large quantities of product may be

purified from fermentation broths. Bacteriocin properties (cationic and hydrophobic nature, low molecular weight) can be exploited for their recovery from complex fermentation broths which contain high levels of peptides (10-30 $g \cdot L^{-1}$) compared to a bacteriocin concentration of 10-100 $mg \cdot L^{-1}$.

Carolissen-Mackay and co-workers (1997) have rewieved protocols for lab-scale purification of bacteriocins from many LAB, namely *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Carnobacterium* and *Lactococcus* spp. The methods reported in this review, mostly used for purification of cationic bacteriocins, generally include a centrifugation step for the removal of cells from the bacterial culture medium, followed by a concentration step. The latter often involves precipitation of peptides with ammonium sulphate and precipitate recovery by centrifugation. The bacteriocin is subsequently purified to homogeneity by one or more chromatographic steps, including cation-exchange, hydrophobic interaction, gel permeation and/or reverse-phase chromatography.

For example, enterocin RJ-11 produced by *Enterococcus faecalis* RJ-11 (Yamamoto et al., 2003) and piscicocin CS526 produced by *Carnobacterium piscicola* CS526 (Yamazaki et al., 2005) were purified to homogeneity by centrifugation, ammonium sulphate precipitation and gel permeation chromatography on Sephadex G50 column, while reverse-phase chromatography was used to purify enterococcin EFS2 (Maisnier-Patin et al., 1996) and enterocin 4 (Joosten et al., 1996).

However, the primary aim of these studies consists in obtaining purified preparations of bacteriocin in order to biochemically characterize them, e.g. determining molecular weight, amino acid sequence, mode of action, etc.. Indeed, these downstream processes are applicable only on small volumes and are characterized by very low overall yield (about 50 % but often less than 30 %). In order to increase the product yield, Guyonnet and co-workers (2000) substituted ammonium sulphate precipitation with cation-exchange chromatography to purify mesentericin Y105 by *Leuconostoc mesenteroides* Y105 broth and other five anti-*Listeria* bacteriocins; however, a purification from a 100 mL culture with a final yield of 60 % took 6 to 8 h.

Uteng and co-workers (2002) proposed a rapid and simple two-step chromatographic procedure suitable for both small- and large-scale purification of class IIa bacteriocins. In the first step the bacterial culture is directly applied on a cation-exchange column; nextly, the bacteriocin fraction is purified on a reverse-phase column. Yield and purity of pediocin PA-1 purified with this method were 80 and 90 %, respectively.

Besides chromatographic steps, other methods to purify bacteriocin use organic solvents. Lactococcin B and pediocin PA-1 were purified in a rapid way by ethanol precipitation, preparative isoelectric focusing and ultrafiltration (Venema et al., 1997). The authors suggested that their method could be applicable with success to small, hydrophobic and cationic bacteriocins.

A sequence of ammonium sulphate precipitation, chloroform/methanol extraction and reverse-phase chromatography was applied to purify amylovorin L471 from *Lactobacillus amylovorus* DCE 471 broth (Callewaert R., 1999). Chloroform/methanol extraction was also used to partially purify seven enterocins by *E. faecium* and *faecalis* strains (Foulquié Moreno et al., 2003), showing as it could be a general method to purify bacteriocins.

Several protocols based on adsorption/desorption have been developed for large scale recovery and purification of bacteriocins. As mentioned above, bacteriocin produced

after growth phase adsorb to the membrane of cell producer. This effect can reduce free bacteriocin available for the subsequent purification (Jack et al., 1995). The pH is a crucial factor in determining the degree of adsorption of the peptide onto cell surface. In general, large amounts (until 90 %) of bacteriocin molecules are adsorbed at pH near 6, whereas the lowest adsorption (< 10 %) took place at pH near 2. On the basis of this information, Yang and co-workers (1992) developed a downstream processing for four bacteriocins (nisin, pediocin AcH, sakacin A and leuconocin Lcm1) adsorbing on the cell surface. It consisted in pasteurizating the culture to kill the producing cells after fermentation, a culture pH adjustment to 6 to allow bacteriocin adsorption, harvesting of cells and their re-suspension in a pH 2 buffer to release the bacteriocin molecules from the cell surface. With this procedure, yields greater than 95 % has been achieved. The resulting material provides a convenient starting point for subsequent bacteriocin purification. However, the recovery may be limited for other strain/bacteriocin combinations (Daba et al. 1994).

AIMS OF THE WORK

The primary aim of this research project was to study bacteriocin production from specific lactic acid bacteria. Particularly, most activity was focused on the development and optimization of bacteriocin production process by an enterococcal strain (*Enterococcus casseliflavus* CGA1) isolated from “salami” meat. In collaboration with a biotech company active in food industry that made us the strain available, we demonstrated that this enterocin has a strong activity towards *Listeria monocytogenes*; therefore it is potentially interesting as food biopreservative.
In order to satisfy this goal, the following topics were planned:

- cost optimization of a growth medium suitable for enterocin production and microorganism growth;
- study of bacteriocin production kinetics in relation to the microorganism growth and metabolism in batch fermentation;
- development of fed-batch fermentative processes for bacteriocin production;
- development of a novel downstream process for purification of the bacteriocin from fermentation broth;
- scale up studies of the process from 2 to 100 liters.

A spectrum activity versus starter cultures/probiotics LAB and different pathogens was also defined.
Moreover, other LAB, used as starter cultures and/or probiotics, were screened for production of bacteriocins active towards pathogens. Namely, the strains *Lactobacillus plantarum* DSM 12028 and *Lactobacillus crispatus* L1 were used in this research work. Their bacteriocin production towards pathogenic microorganisms was studied in flask and batch fermentations.

CHAPTER II

2. MATERIALS AND METHODS

2.1 Materials

Growth media components, ammonium sulphate and ethylene diamino tetraacetic acid (EDTA) were supplied by Sigma Aldrich (USA).
Tryptic Soy Broth (TSB), bacteriological agar and de Man-Rogosa-Sharp (MRS) medium were purchased by Oxoid (United Kingdom).
Vegetable peptone E1, soy peptone A2SC, soy peptone E110, wheat peptone E430, meat extract and yeast extract were supplied by Organochemie (France).

2.2 Strains and media

The enterococcal strain used in this work was *Enterococcus casseliflavus* CGA1, isolated from industrial "salami" production by an Italian company and characterized by API 20 NE bioMerieux (France) in our department. The other LAB tested for bacteriocin production were probiotic strains *Lactobacillus plantarum* DSM 20128 purchased by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmb H, Germany) and *Lactobacillus crispatus* L1, human isolated by vaginal secretions and previously characterized by API 20 NE bioMerieux.
The producing strains were stored on agar plates at 4 °C and in a 20% v/v glycerol solution at -80 °C.
The basal medium, whose formulation was also suggested by the industry, used for *E. casseliflavus* CGA1 growth is shown in Table 5. The salts stock solution, stored at 4 °C, was composed as follows: $MgSO_4 \cdot 7H2O$, 20 $g \cdot L^{-1}$; $MnSO_4 \cdot H2O$, 5 $g \cdot L^{-1}$; $CaCl_2 \cdot 2H_2O$, 10 $g \cdot L^{-1}$; $FeSO_4 \cdot 7H_2O$, 2 $g \cdot L^{-1}$; $CuSO_4 \cdot 5H_2O$, 0.1 $g \cdot L^{-1}$; $CoCl_2 \cdot 6H_2O$, 0.08 $g \cdot L^{-1}$; $ZnSO_4 \cdot 7H_2O$, 0.8 $g \cdot L^{-1}$; Na_2MoO_4, 0.08 $g \cdot L^{-1}$; EDTA·2Na, 35 $g \cdot L^{-1}$.
The vitamin solution was prepared re-suspending one tablet of commercially obtained BeTotal in 100 mL of deionised water, filtered on 0.22 μm (Millipore, USA) and stored at 4 °C.
L. plantarum DSM 12028 and *L. crispatus* L1 were grown on a semi-defined medium (Kimmel and Roberts, 1998), eventually modified in the concentration of bactocasitone (Difco, France) as previously described by Schiraldi and co-workers (2003).
The microorganisms used as indicators to assay bacteriocin activity, and their growth media and temperatures are reported in Table 6.
Medium components were thermally sterilized in autoclave (120 °C for 15 min) except glucose and vitamin solution that were filtered on 0.22 μm filter devices (Millipore, USA) and added afterwards.

2.2.1 Agar plates preparation

To maintain the producing strains, agar plates were prepared using MRS medium with the addition of 20 $g \cdot L^{-1}$ of agar. Petri dishes were stricken with a cell suspension and then were incubated at 37 °C overnight (except *L. plantarum* DSM 12028 that was grown at 30 °C) and stored at 4 °C if necessary.

Table 5
Basal growth medium for *E. casseliflavus* CGA1.

Nutrient	Concentration
Glucose	20 $g \cdot L^{-1}$
N complex source	20 $g \cdot L^{-1}$
Ammonium citrate	13 $g \cdot L^{-1}$
NaCl	6 $g \cdot L^{-1}$
KH_2PO_4	2 $g \cdot L^{-1}$
Salts	20 $mL \cdot L^{-1}$ (from stock solution)
Complex B vitamins	2 $mL \cdot L^{-1}$ (from stock solution)

Table 6
Indicator microorganisms used in this work.

Indicator microorganims	Growth medium	Temperature (°C)
Enterococcus faecium F1	Tryptic Soy Broth	30
Lactobacillus casei DSM 20011	MRS broth	37
Lactobacillus bulgaricus DSM 21081	MRS broth	37
Lactobacillus crispatus L1	MRS broth	37
Lactococcus lactis NZ9000	M17 broth	30
Lactobacillus plantarum DSM 12028	MRS broth	30
Escherichia coli BL21	Luria-Bertani medium	37
Escherichia coli K4	Luria-Bertani medium	37
Listeria monocytogenes	Tryptic Soy broth	37
Staphylococcus aureus	Luria-Bertani medium	37
Streptococcus agalactiae	Luria-Bertani medium	37
Klebsiella pneumoniae	Luria-Bertani medium	37

2.3 Flask experiments

Flasks experiments, performed in order to optimize the growth medium for *E. casseliflavus* CGA1, were carried out in a rotary air shaker (model Minitron, Infors, Switzerland) at 37°C, with a rotation speed of 50 rpm using 250 mL flasks. The medium/shake flask volume ratio was kept equal to 1.6. The medium was inoculated with a glycerol stock solution and growth kinetics, like in the other experiments, was evaluated by the determination of the absorbance at 600 nm (Spectrophotometer DU800, Beckman Coulter, USA).
At regular time intervals (generally 1 hour), samples were collected, centrifuged at 1700xg (centrifuge model: Avanti J-20 XP, Beckman Coulter, USA), and tested for bacteriocin activity.
Flask experiments were also performed to study the effect of medium on the bacteriocin production from *L. plantarum* DSM 12028 and *L. crispatus* L1. They were carried out through the procedure described above.

2.4 Fermentation experiments

For fermentation experiments, three fermenters (Biostat CT, Biostat C and Biostat D; Braun Biotech International, Germany) with different volume were used.
The Biostat CT is a fermenter with a working volume of 2 L; the Biostat C has a working volume of 15 L; the Biostat D working volume is 100 L. They are *in situ* sterilizable and connected to a DCU (Digital Control Unit) to set and control different parameters as pH, pO_2, stirring speed (rpm), air flow rate ($L \cdot min^{-1}$) and temperature (°C). Moreover, the fermenters were connected to a PC and remotely controlled by the MFCS-win software. The systems were provided with four peristaltic pumps for the addition of alkali, acid and nutrients, and with temperature, pH and pO_2 probes.
Each fermentation experiment was performed on triplicate.

2.4.1 Batch fermentations

Batch experiments performed with *E. casseliflavus* CGA1 were first carried out in the 2 L Biostat CT described above. 2 L of optimized medium in the fermenter were inoculated from seed cultures overnight grown so that the starting absorbance (600 nm) value was kept between 0.08 and 0.12. The pH was always set and kept constant at 7 by adding NH_4OH 30 % (v/v) or H_2SO_4 30 % (v/v). The temperature and stirring speed were also kept constant at 37 °C and 50 rpm, respectively. The fermentations were performed without aeration.
During the cultivations, 5 mL samples were withdrawn from the reactor at regular time intervals for the determination of absorbance at 600 nm, bacteriocin activity, glucose and lactic acid concentrations. Using a conversion factor of 0.52, OD values were converted in dry biomass concentrations (X, $g \cdot L^{-1}$), as previously reported for lactic acid bacteria (Schiraldi et al., 2003).
Batch experiments were reproduced, in scaling up experiments, on Biostat C and Biostat D fermenters. Culture volumes were 15 and 100 L, respectively. The process conditions (temperature, pH, stirring) were the same.
Batch experiments on 2 L scale were also performed to assay the bacteriocin production

from *L. plantarum* DSM 12028 and *L. crispatus* L1. 2 L of appropriate medium were inoculated using the procedure described above. The pH was kept constant at 6.5 for both microorganisms, while the temperature was 30 °C for *L. plantarum* DSM 12028 and 37 °C for *L. crispatus*. Stirring was set at 100 rpm. The fermentations were performed without aeration. Samples (5 mL) were regularly withdrawn from the fermenter for the determination of absorbance at 600 nm and bacteriocin activity.

2.4.2 Fed-batch fermentations

Fed-batch experiments for *E. casseliflavus* CGA1 were firstly carried out on the 2 L fermenter. Two different fed-batch strategies (pulse and continuous feeding) were implemented.
In the pulse feeding experiments, the batch phase last 5 h, then pulses of fresh concentrated medium were done at 5, 7 and 9 h. The medium was concentrated in order to give, after the addition in the culture volume, the initial concentrations of each component.
In the continuous feeding experiments, after 5 h of batch phase, a fresh concentrated medium was fed into bioreactor. The feeding strategy was designed according to the following equations:

$$F = \frac{XVq_S}{S_i} \qquad \text{(eq. 1)}$$

$$q_S = \mu Y_{xs} \qquad \text{(eq. 2)}$$

In these equations, F is the feed flow rate ($L \cdot min^{-1}$), X is the initial biomass concentration ($g \cdot L^{-1}$), V is the starting colture volume (L), q_s is the specific substrate consumption ($g \cdot g^{-1} \cdot h^{-1}$), S_i is the limiting substrate concentration in the feeding solution ($g \cdot L^{-1}$), μ is the specific growth rate (h^{-1}), and $Y_{X/S}$ is the yield coefficient of biomass on substrate ($g \cdot g^{-1}$). In our experiments, glucose (carbon and energy source) was the limiting substrate. F was set hour per hour from glucose consumption using these equations.
The concentrated feeding solution contained 200 $g \cdot L^{-1}$ of glucose, 25 $g \cdot L^{-1}$ of soy peptone A2SC, and the other nutrients at the concentrations reported in Table 5.
For the duration of all cultivations, 5 mL samples were withdrawn from the reactors at regular time intervals.
Only the fed-batch experiments based on continuous feeding strategy were reproduced on 15 and 100 L fermenters, to test the scalability of the process.

2.5 Downstream processes

At the end of fed-batch fermentations, 2 L culture broth was centrifuged at 5000xg at 4 °C for 40 min (centrifuge model: Avanti J-20 XP, Beckman Coulter, USA) and the supernatants were collected and submitted to different downstream procedures.

2.5.1 Purification procedure with buthanol/acetone

The supernatant was acidified at pH 1.5 with 1 M HCl. 200 mL of buthanol and 600 g of NaCl were added; the extraction with buthanol was performed for 3 h under stirring. The phase separation was carried out by a separating funnel. The organic phase (1/10 of the initial volume) was collected, while the water one was extracted again (1.5 h), after adding of 100 mL of buthanol; the second organic phase was added to the first one. 500 mL of acetone were added; the precipitation with acetone was performed for 2 h, at 4 °C, under stirring. The wet solid was recovered by filtration using a filter cloth and dried under vacuum, for 4 h, at 40 °C. At the end of each phase, samples were collected and tested for bacteriocin activity and protein concentration. All reagents for this procedure were purchased by Sigma-Aldrich.
This procedure is schematically represented in figure 2(a).

2.5.2 Purification procedure with methanol/chloroform

The enterocin was purified by lightly modifying the procedure reported by Callewaert and co-workers (1999).
Supernatant pH was adjusted to 6.5 with 2 M NaOH. This cell-free culture supernatant was brought to a final ammonium sulphate concentration of 40 % saturation by slow addition of the salt, and was stirred overnight at 4 °C. Then, the mixture was centrifuged (5000xg, 30 min, 4 °C) and the bottom pellets and the surface pellicles were harvested and resuspended in 200 mL of 50 mM sodium phosphate buffer (pH 6.5). To one volume of the resuspended product, 15 volumes of a methanol-chloroform mixture (1:2, v/v) were added, and the mixture was extracted at 4 °C for 1 h. The resulting fine-grained white precipitate (containing the product) was collected by centrifugation (5000xg, 30 min, 4 °C) and air dried. This partially wet precipitate was dried under vacuum, for 4 h, at 40 °C. At every step, samples were collected and tested for bacteriocin activity and proteins concentration. The reagents used were purchased by Sigma-Aldrich.
The procedure described is schematically outlined in figure 2(b).

2.5.3 Purification procedure based on membranes

2.5.3.1 Description of equipment

In order to perform and to study the enterocin purification through ultrafiltration membranes on 2 liters scale, tangential flow filtration ÄKTAcrossflow system (GE Healthcare, USA) was used. It is a completely automatized system to perform microfiltration/ultrafiltration operations using hollow fibers or cassettes cartridges with filtering area from 0.004 to 0.02 m^2. The system is equipped with a sample reservoir of

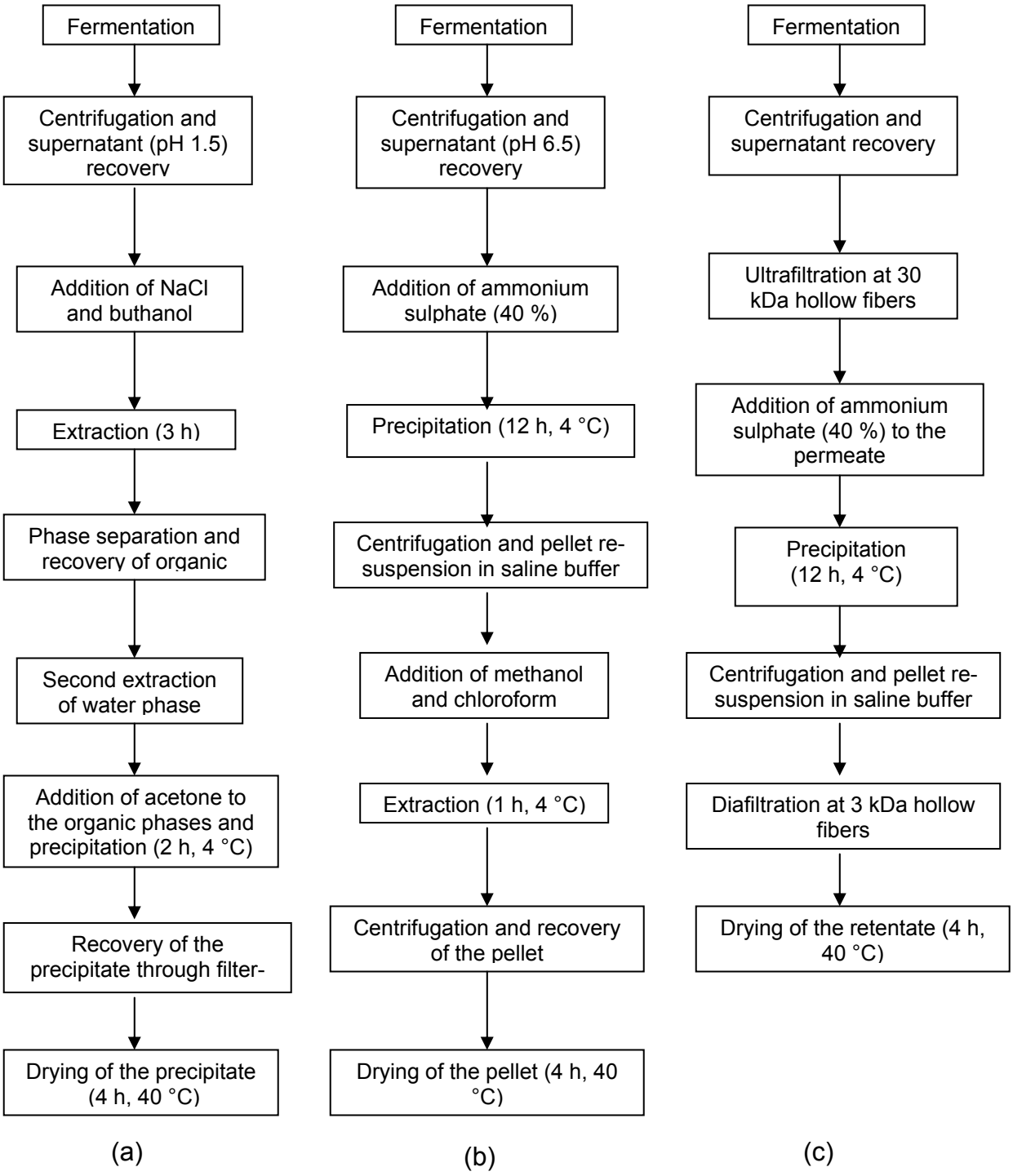

Figure 2
Schematic representation of the three enterocin downstream processes compared in this work. (a) buthanol/acetone procedure; (b) methanol/chloroform procedure; (c) purification based on membranes.

350 mL, a transfer pump (0-200 mL·min^{-1}) for inlet of process sample, diafiltration buffer, and CIP (Cleaning In Place) solutions; a feed pump (0-600 mL·min^{-1}) for feeding of the sample from the reservoir to the filter and recirculation to the reservoir; a permeate pump (0-600 mL·min^{-1}) to generate a back-pressure for fine TMP regulation. Moreover, it is equipped with a sensor level to perform fed-batch concentration or constant volume diafiltration; temperature probe on the retentate line; pressure sensors and flow meters on feed, retentate and permeate lines; and pHmeter, conductivity meter and UV (with λ=280 nm) detector on permeate line. It is connected to a PC and a UNICORN^{TM} software, that allows to monitor and control the process and its parameters.

2.5.3.2 Purification procedure with membranes

The cell-free supernatant was ultrafiltered using 30 kDa hollow fiber cartridges housed on ÄKTAcrossflow. The membranes had a surface area of 0.014 m^2 and an inner diameter of 0.5 mm. During the process, retentate flow rate and TMP were set constant at 150 mL·min^{-1} (cross flow rate=10.7 lpm·m^{-2}) and 1 bar, respectively. The final concentration factor of the sample and the diafiltration exchange factor were 10 and 3, respectively. Ultrapure water (MilliQ; Millipore, USA) was used as diafiltration buffer. The permeate was collected and brought to a final ammonium sulphate concentration of 40 % saturation by slow addition of the salt, and was stirred overnight at 4 °C. Then, the mixture was centrifuged (5000xg, 30 min, 4 °C) and the bottom pellets and the surface pellicles were harvested and resuspended in 200 mL of 50 mM sodium phosphate buffer (pH 6.5). This volume was 2-fold concentrated and diafiltered to remove salts using 3 kDa hollow fiber cartridges housed on ÄKTAcrossflow. The membranes had a surface area of 0.014 m^2 and an inner diameter of 0.5 mm. During the process, retentate flow rate and TMP were set constant at 200 mL·min^{-1} (cross flow rate=14.2 lpm·m^{-2}) and 1.25 bar, respectively. The diafiltration exchange factor to obtain a conductivity below 100 μS·cm^{-1} was 10. Ultrapure water (MilliQ; Millipore, USA) was used as diafiltration buffer. The retentate was collected and dried under vacuum, for 4 h, at 40 °C. For every fraction of the purification process, samples were collected and tested for bacteriocin activity and proteins concentration.
This purification procedure is schematically resumed in figure 2(c).

2.5.3.3 Optimization of 3 kDa ultrafiltration process

The optimal values of cross flow rate and transmembrane pressure for the concentration and diafiltration process on 3 kDa hollow fibers were determined through appropriate experiments using ÄKTAcrossflow.
The system was put in complete recirculation modality (with both retentate and permeate lines returning to the reservoir). 300 mL of sample were filtered scouting five different TMPs (0.5, 1, 1.5, 2 and 2.5 bar) at a retentate flow of 200 mL·min^{-1}. The same experiment was repeated at a retentate flow of 125 mL·min^{-1} and 50 mL·min^{-1}, respectively. In each trial, the instrument recorded the flux value at every TMP.
Plots of flux versus TMP at the different retentate flows were built and used to find the optimal conditions to perform the process.

2.5.3.4 Scale-up of the membrane purification procedure

The purification through membranes was also performed on 10 L scale, to demonstrate the scalability of the process. The tangential flow filtration system used was the UniFlux10 (GE Healthcare, USA). It is a completely automatized pilot system to perform microfiltration/ultrafiltration operations using hollow fibers or cassettes cartridges with filtering area from 0.1 to 1 m^2. The system is equipped with a sample reservoir of 10 L, a buffer feed pump for inlet of process sample, diafiltration buffer, and CIP (Cleaning In Place) solutions; and a membrane feed pump (0-10 $L \cdot min^{-1}$) for feeding of the sample from the reservoir to the filter and recirculation to the reservoir. Moreover, it is equipped with a sensor level to perform fed-batch concentration or constant volume diafiltration; temperature probe on the retentate line; pressure sensors and flow meters on feed, retentate and permeate lines; and pH meter, conductivity meter and UV (with λ=280 nm) detector on permeate line. It is connected to a PC and a UNICORN^{TM} software, that allows to control, manage and monitor the process and its parameters.
In order to scale up the 30 and 3 kDa ultrafiltration processes, hollow fibers with 0.2 m^2 filtering area and 0.5 mm inner diameter were used. The process conditions (cross flow rate and TMP) for both processes were the same used and optimized on ÄKTAcrossflow.

2.6 Analytical methods

2.6.1 Bacteriocin activity assay

Samples collected during fermentation experiments of *E. casseliflavus* CGA1, *L. plantarum* DSM 12028 and *L. crispatus* or purification procedures of *E. casseliflavus* CGA1 broth were tested for bacteriocin activity by agar well diffusion assay and/or critical dilution assay (Parente et al., 1995).
Twenty mL of the appropriate agar (1.5 % w/v) medium were pipetted in 90 mm Petri dishes and inoculated with a standardized indicator suspension growth overnight. The inoculum volume was chosen so that the absorbance at 600 nm was 0.1. After drying for 30 min, four 8 mm wells were bored in each plate. 100 μl of the sample not diluted (for agar well diffusion assay) or 2-fold dilutions of each sample (for critical dilution assay) were pipetted into each well and the plates were incubated overnight at a temperature depending on the indicator strain. For the first method, the diameter of the inhibition zones was measured using a calliper. For the second one, that gives a more quantitative measure, the plates were checked for inhibition zones. The critical dilution was defined as the highest dilution that produce a definite inhibition zone. When a clear inhibition zone was followed by a hazy one, the critical dilution was calculated a the average of the two dilutions. The titer of the bacteriocin solution, expressed in $BU \cdot mL^{-1}$, was calculated as (1000/d) x D, where D is the critical dilution and d the dose (the amount of bacteriocin solution pipetted in each spot; 100 μL in our case). For growth experiments, bacteriocin specific production was also calculated as bacteriocin activity per biomass unit and expressed as $BU \cdot mL^{-1} \cdot OD^{-1}$.
Agar well diffusion assay was used to test bacteriocin activity in order to define spectrum activity. This spectrum comprised the microorganism reported in Table 6. The inhibitory

activity versus *E. coli* strains was assayed in presence of 0.005 $g \cdot l^{-1}$ of EDTA.
Critical dilution assay was used in the other cases. When this test was performed, *Enterococcus faecium* F1 was always used as indicator strain to test the bacteriocin activity of *E. casseliflavus* CGA1, while *Staphilococcus aureus* was the indicator test for *L. plantarum* DSM 12028 and *L. crispatus* L1.

2.6.2 Determination of enterocin molecular weight

The molecular weight range of the enterocin was determinated as follows. A 4 mL surnatant sample obtained by the growth of *E. casseliflavus* CGA1 was ultrafiltered using 10 kDa centrifugal filter devices (YM-10 Centricon; Millipore, USA) at 5000xg. The filtrate (3.5 mL) was splitted in a retentate (0.5 mL) and a further filtrate (3 mL) through ultrafiltration using 3 kDa centrifugal filters devices (YM-3 Centricon). Each fraction was diluted with deionised water to initial volume (4 mL). The retentates over 10 and 3 kDa and the filtrate under 3 kDa were tested for bacteriocin activity.
Triplicate experiments were carried out.
To estimate the purity after purification and to determinate more exactly the molecular weight of the enterocin, tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) was carried out as described by Schägger and von Jagow (1987). Polyacrylamide concentrations in the stacking and separating gels were 9.6 and 16 %, respectively. Electrophoresis was conducted at a constant voltage of 30 V for 1 h and 90 V for 12 h. The following standard proteins (Amersham, UK) were used: insulin α chain (2300 Da), insulin β chain (3400 Da), bovine trypsin inhibitor (6200 Da), lysozyme (14300 Da), β-lactoglobulin (18400 Da), carbonic anhydrase (29000 Da), and ovalbumin (43000 Da). After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. SDS, tricine and Coomassie were purchased by Sigma Aldrich (USA).

2.6.3 Determination of glucose, lactic acid and protein concentrations

Samples collected during fermentation experiments were centrifuged and supernatants were successively ultrafiltered using 10 KDa centrifugal filter devices (YM-10 Centricon) at 5000xg. The filtrate was employed for the quantification of consumed glucose and produced lactic acid.
Glucose concentration was determined through HPAEC-PAD analysis performed with a chromatographer (model ICS-3000, Dionex, USA) using a Carbopac PA100 column (Dionex) and a pulsed amperometric detector (reference electrode Ag-AgCl; measure electrode Au). Components separation was performed by a gradient elution using 160 mM NaOH and 400 mN CH_3COONa as eluents with a flow rate of 1 $mL \cdot min^{-1}$. For quantification, a method based on external standards was used.
Lactic acid and other organic acids present in the culture broth were analysed by isocratic elution with 0.1 % (v/v) phosphoric acid solution, on a Supelcogel TM C610H column (Supelco, USA) equipped with precartridge Supelgard C610H at 32 °C, using a Dionex-HPLC supplied with a P580 pump. Detection was carried out with a UV-detector 170S at λ=210 nm. The flow rate was 0.5 $mL \cdot min^{-1}$. For quantification, a method based on external standards was used; the standards solution was composed by lactic, citric,

butyrric, isobutyrric, acetic and formic acids.
Protein concentration was determined through the Bradford assay (Bradford, 1976) using a calibration curve obtained with BSA standard solutions. The reagents were purchased by Bio-Rad Laboratories (Germany).
In the downstream processes, the increase in the bacteriocin specific activity (expressed as $BU \cdot mg^{-1}$) was calculated from antimicrobial activity ($BU \cdot mL^{-1}$) and proteins concentration ($mg \cdot mL^{-1}$).

CHAPTER III

3 RESULTS

3.1 Flask experiments

In order to formulate a growth medium suitable for bacteriocin production from *Enterococcus casseliflavus* CGA1, flask experiments were performed. The medium composition, used as base for the medium development in all experiments, is reported in Table 5, except when MRS was used: in this case, it completely replaced this medium.
Two kinds of flask experiments were performed: first, the best complex nitrogen source for growth and bacteriocin production was found; second, the effect of decrease of carbon and nitrogen source concentrations was tested.
Flask experiments were finally carried out to study the effect of nitrogen complex source on bacteriocin production by *L. plantarum* DSM 12028 and *L. crispatus* L1.

3.1.1 Determination of the nitrogen complex source

Vegetable peptone E1, soy peptone A2SC, soy peptone E110, wheat peptone E430, meat extract, yeast extract and MRS were tested in order to find the optimal complex nitrogen source for enterocin production from *E. casseliflavus* CGA1. The nutrient concentration (20 $g \cdot L^{-1}$) was the same in all experiments, except MRS, concentrated at 52 $g \cdot L^{-1}$.
In Table 7 the results of growth experiments, in terms of optical density (OD) at 600 nm, bacteriocin activity, and bacteriocin specific production (calculated as $BU \cdot mL^{-1} \cdot OD^{-1}$) are summarized; bacteriocin activities are calculated at 8 h, while optical densities at 8 and 24 h.
In terms of microorganism growth, the best data were obtained using soy peptone E110 as nitrogen source. An absorbance of 4.5 was obtained after 8 h of growth; this value is kept constant through out the experiments, indicating the starting of stationary phase after 8 h.
A similar trend was obtained for other experiments. Particularly, the growth with soy peptone A2SC and yeast extract was almost the same, being characterized by a final OD of 4.1 and 3.9, respectively. Similarly, the growth in MRS, vegetable peptone E1 and wheat peptone E430 gave very close OD values. Only using malt extract as complex nitrogen source, the biomass production was very low, with OD values of 0.9 (8 h) and 1.1 (24 h). As far as the bacteriocin production is concerned, a maximum of activity was obtained for every culture after 8 h of growth, except when the microorganism was supplied of malt extract; in this case none bacteriocin production was detected.
The best bacteriocin activity (80 $BU \cdot mL^{-1}$) was obtained in MRS, soy peptone A2SC and soy peptone E110. In terms of specific production (that gives the bacteriocin concentration produced per biomass unit), despite of a lower biomass production, utilization of soy peptone A2SC as nitrogen source allowed to obtain 20.5 $BU \cdot mL^{-1} \cdot OD^{-1}$, higher than the value obtained in the culture containing soy peptone E110. For this reason, soy peptone A2SC was selected as nitrogen source for the next growth experiments. A high specific production at 8 h (22.7 $BU \cdot mL^{-1} \cdot OD^{-1}$) was also detected when the microorganism was grown in MRS.

Table 7
Comparison of growth parameters and bacteriocin activities in media containing different nitrogen complex sources.

Nutrient	OD (8 h)	OD (24 h)	BU·mL^{-1} (8 h)	BU·mL^{-1}·OD^{-1} (8 h)
Vegetable peptone E1	3.5	3.6	40	11.2
Soy peptone A2SC	3.8	4.1	80	20.5
Soy peptone E110	4.5	4.3	80	17.7
Wheat peptone E430	3.4	3.5	40	11.4
Yeast extract	3.8	3.9	60	15.4
Malt extract	0.9	1.1	0	0
MRS	3.5	3.5	80	22.7

Data of bacteriocin activity are in triplicates with standard deviation included between 5-10 %.

3.1.2 Effect of the carbon and nitrogen source concentrations

Once selected the nitrogen complex source, flask experiments were also carried out to test the influence of reducing the concentration of carbon (glucose) and nitrogen (soy peptone A2SC) sources on the microorganism growth and enterocin activity. The basal concentration for both nutrients is 20 $g \cdot L^{-1}$ (Table 5). The microorganism growth was tested in media containing decreased concentrations for both nutrients, until to 5 $g \cdot L^{-1}$.
Figure 3 shows the comparison of growth curves. As expected, the decreasing of nutrient concentrations, particularly the nitrogen source, has effect on biomass production. In fact, in media containing 20 and 10 $g \cdot L^{-1}$ of soy peptone A2SC, the final OD, measured at 24 h, ranges from 4.1 to 3.8, depending on the glucose concentration. A lower biomass production is obtained when the microorganism is grown with soy peptone A2SC 5 $g \cdot L^{-1}$, independently on the glucose concentration. Particularly, after 8 h of growth, the absorbance is very similar (about 2.8) for each experiments, while a slight decrease is observed for the culture containing 5 $g \cdot L^{-1}$ of both nutrients.
The bacteriocin activity at 8 h was also detected in these cultures (Table 8). Interestingly, very similar bacteriocin activity was obtained, although the differences observed in the biomass production. An enterocin activity of 80 $BU \cdot mL^{-1}$ was detected when soy peptone A2SC concentration was 10 or 20 $g \cdot L^{-1}$, while 60 $BU \cdot mL^{-1}$ were found in cultures grown with soy peptone A2SC 5 $g \cdot L^{-1}$. Despite this slight difference, higher values of specific production were obtained in poorer media. In particular, specific production of 22.9 $BU \cdot mL^{-1} \cdot OD^{-1}$ was measured for the culture grown in the medium containing 5 $g \cdot L^{-1}$ of glucose and soy peptone A2SC. For this high enterocin production per biomass unit, this medium was chosen as suitable for the following fermentations experiments.

3.1.3 Flask experiments: *L. plantarum* DSM 12028 and *L. crispatus* L1

In order to evaluate the effect of different bactocasitone concentrations on bacteriocin production by *L. plantarum* DSM 12028 and *L. crispatus* L1, flask experiments were also performed. Concentrations of 1, 5 and 10 $g \cdot L^{-1}$ of complex nitrogen source were tried. Table 9 reports the data obtained in terms of growth and bacteriocin production. For *L. plantarum* DSM 12028, the reduction of bactocasitone concentration from 10 to 5 $g \cdot L^{-1}$ did not influence the growth and bacteriocin production: at 8 h, 40 $BU \cdot mL^{-1}$ were detected for both cultures. Bacteriocin specific production was generally similar.
Differently from *L. plantarum* DSM 12028, the reduction of bactocasitone concentration from 10 to 5 $g \cdot L^{-1}$ in *L. crispatus* L1 cultivations determined a decrease of bacteriocin activity from 40 to 20 $BU \cdot mL^{-1}$, respectively.
Lower biomass production (2.2 OD for *L. plantarum* DSM 12028 and 1.6 for *L. crispatus* L1) and no bacteriocin activity were detected in the experiments with 1 $g \cdot L^{-1}$ of bactocasitone.

3.2 Fermentations experiments in batch modality

3.2.1 Batch fermentations of *E. casseliflavus* CGA1

Batch fermentations were performed to test the *E. casseliflavus* CGA1 growth on the

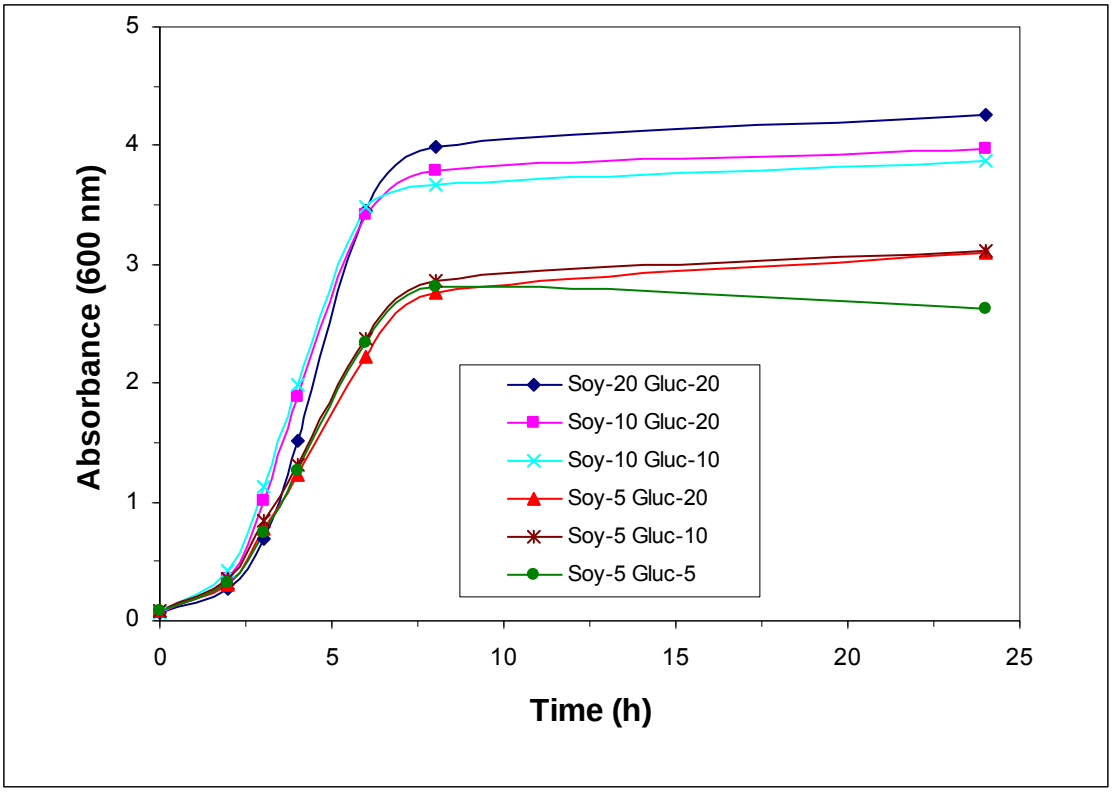

Figure 3
Comparison of curves obtained growing *E. casseliflavus* CGA1 in media containing different concentrations of soy peptone A2SC and glucose ($g \cdot L^{-1}$).

Table 8
Comparison of the bacteriocin activities of the cultures in figure 3.

Medium	BU·mL^{-1} (8 h)	BU·mL^{-1}·OD^{-1} (8 h)
Soy-20/Gluc-20	80	20.6
Soy-10/Gluc-20	80	21.1
Soy-10/Gluc-10	80	21.6
Soy-5/Gluc-20	60	23.0
Soy-5/Gluc-10	60	22.6
Soy-5/Gluc-5	60	22.9

Data of bacteriocin activity are in triplicates with standard deviation included between 5-10 %.

Table 9
Growth and bacteriocin activities of *L. plantarum* CGA1 and *L. crispatus* in flask and batch fermentations.

Microrganism	Scale	Bactocasitone concentration ($g \cdot L^{-1}$)	OD (24 h)	$BU \cdot mL^{-1}$	$BU \cdot mL^{-1} \cdot OD^{-1}$
L. plantarum DSM 12028	Flask	10	3.8	40	10.4
L. plantarum DSM 12028	Flask	5	3.6	40	11.0
L. plantarum DSM 12028	Flask	1	2.2	0	0
L. crispatus L1	Flask	10	2.6	40	15.3
L. crispatus L1	Flask	5	2.0	20	9.8
L. crispatus L1	Flask	1	1.6	0	0
L. plantarum DSM 12028	Batch	5	8.3	120	14.4
L. crispatus L1	Batch	10	6.0	80	13.3

Data of bacteriocin activity are in triplicates with standard deviation included between 5-10 %.

optimized medium at a higher scale and to characterize the growth parameters, in terms of specific growth rate (μ, h^{-1}) and yield coefficients Y_{xs} (yield of biomass on substrate, g of biomass·g of substrate^{-1}) and Y_{ps} (yield of product on substrate, g of product·g of substrate^{-1}). The kinetics of enterocin and lactic acid production, and glucose consumption, were also characterized.
Experiments were initially performed on the 2 L fermenter (Biostat CT). The comparison of growth curve and kinetics of glucose consumption and lactic acid production is reported in figure 4. From the plot you can see that the exponential phase ended after 6 h of fermentation, when the OD value is 2.9, that corresponds to a biomass concentration of 1.5 g·L^{-1}. A constant biomass concentration (stationary phase) was kept for the remaining fermentation time, with a little decrease detected at 24 h (X=1.4 g·L^{-1}). The μ calculated for the exponential phase was 0.65 h^{-1}, corresponding to a duplication time (t_d) of 1.06 h.
As expected the glucose was completely consumed during the exponential phase, decreasing averagely from 5 g·L^{-1} at 0 h to 0 g·L^{-1} at 8 h. The Y_{xs}, calculated for this phase, is 0.22 g·g^{-1}. As the glucose is consumed, lactic acid is produced as metabolic product of the glucose fermentation. After 6 h of fermentation, about 5 g·L^{-1} of lactic acid were produced with an average Y_{ps} of 0.83 g·g^{-1}, demonstrating the homolactic behaviour of this microorganism.
The bacteriocin activity was also evaluated in batch fermentations and the kinetics of production was characterized. In figure 5 the batch growth curve is compared with the activity of the enterocin, expressed in BU·mL^{-1}. The plot shows how the peptide was produced during the exponential phase, thus reaching a maximum of 80 BU·mL^{-1} at the end of the active growth phase. Constant levels of product were kept as the culture is in early stationary phase. At 24 h, a decrease of activity to 60 BU·mL^{-1} was observed.

3.2.2 Scale up of the batch experiments

In order to evaluate the scalability and the reproducibility of the process on larger scales, batch fermentations were carried out on a 15 L fermenter (Biostat C) and a 100 L fermenter (Biostat D). The same conditions, in terms of medium composition, pH, stirring and temperature, were used.
Table 10 reports the fermentation parameters and the bacteriocin activities for the three different scales. Only small variability can be found between the three fermentation processes. Particularly, the highest final biomass production was obtained in the intermediate scale (1.4 g·L^{-1}), despite the lowest specific growth rate (0.63 h^{-1}). Values of yield coefficients were comparable, ranging from 0.22 to 0.24 g·g^{-1} for Y_{xs} and from 0.79 to 0.88 g·g^{-1} for Y_{ps}.
Interestingly, also the bacteriocin activities were very close. 15 and 100 liters cultures could produce 60 and 80 BU·mL^{-1} at 6 h, respectively. A decrease of bacteriocin activity was detected in late stationary phase (24 h) for both fermentations as well as for the small scale one. In particular, a sharp decrease (from 80 to 30 BU·mL^{-1}) was observed for the larger scale cultivations.

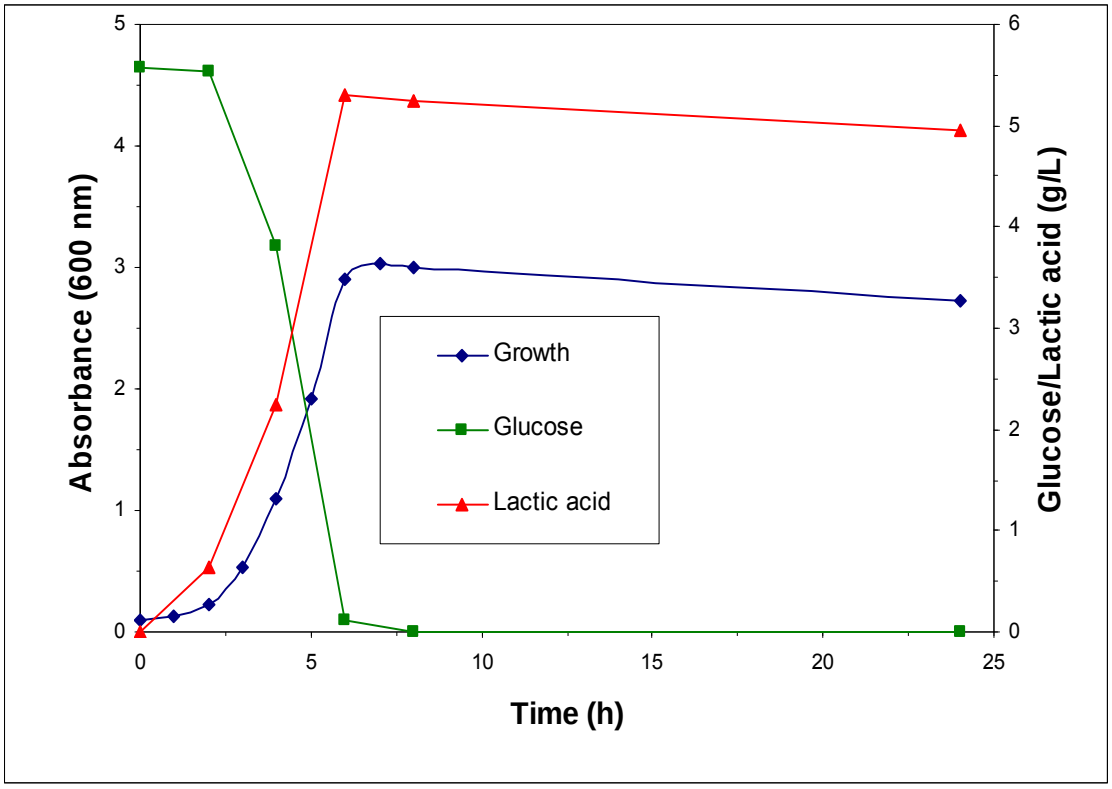

Figure 4
Comparison of the growth curve for *E. casseliflavus* CGA1 in batch fermentation with glucose consumption and lactic acid production.

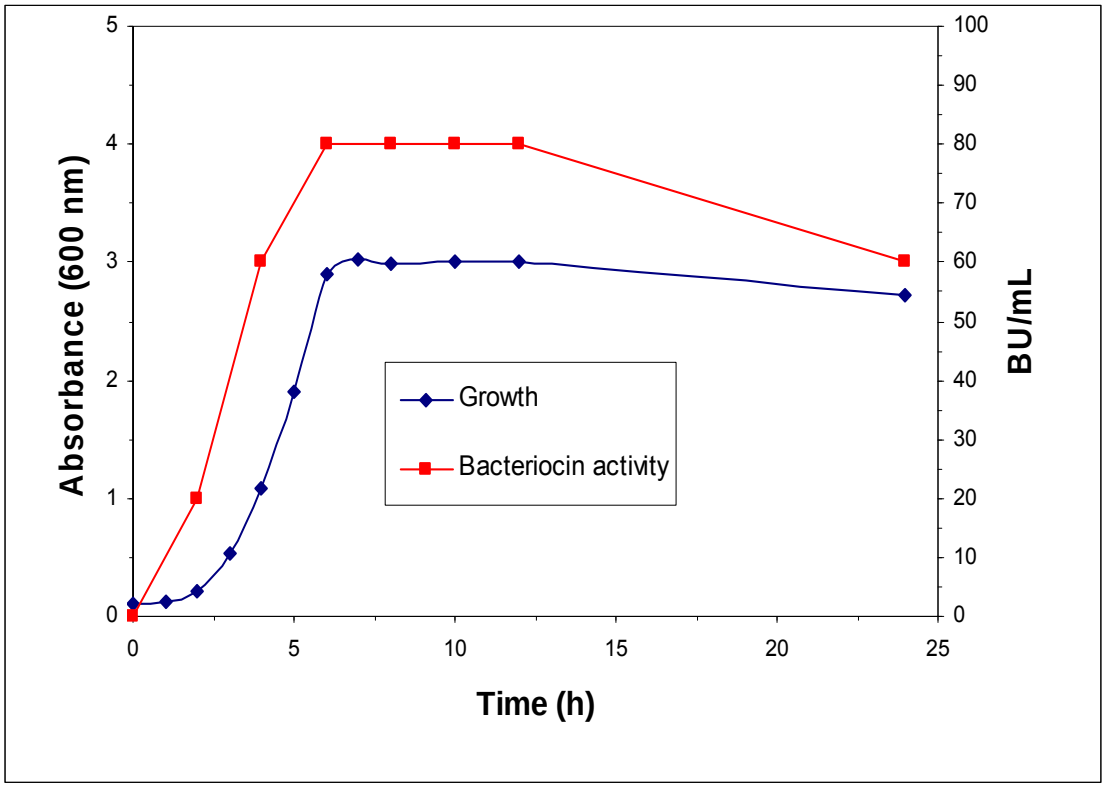

Figure 5
Growth curve of *E. casseliflavus* CGA1 in batch fermentation and enterocin production profile.

Table 10
Comparison of the batch fermentations on 2, 15 and 100 liters.

Culture volume (Fermenter)	Final X ($g \cdot L^{-1}$)	μ (h^{-1})	Y_{xs} ($g \cdot g^{-1}$)	Y_{ps} ($g \cdot g^{-1}$)	BU·mL^{-1} (6 h)	BU·mL^{-1} (24 h)
2 L (Biostat CT)	1.38	0.65	0.22	0.83	80	40
15 L (Biostat C)	1.42	0.63	0.24	0.88	60	40
100 L (Biostat D)	1.39	0.68	0.23	0.79	80	30

Data of bacteriocin activity are in triplicates with standard deviation included between 5-10 %.

3.2.3 Batch fermentations of *L. plantarum* DSM 12028 and *L. crispatus* L1

Batch fermentations of *L. plantarum* DSM 12028 and *L. crispatus* L1 were carried out in order to study the bacteriocin production in a more controlled environment compared to flasks. The concentration of bactocasitone was 5 $g \cdot L^{-1}$ for *L. plantarum* DSM 12028 and 10 $g \cdot L^{-1}$ for *L. crispatus* L1 fermentation.
Table 9 summarizes the data obtained. In comparison with flask experiments, a significative improvement in terms of bacteriocin production was detected for *L. plantarum* DSM 12028: 120 $BU \cdot mL^{-1}$ were obtained after 8 h of growth. It corresponds to a specific bacteriocin production of 14.4 $BU \cdot mL^{-1} \cdot OD^{-1}$. For *L. crispatus* L1 batch fermentation allowed to double the bacteriocin activity (80 $BU \cdot mL^{-1}$).

3.3 Fermentation experiments in fed-batch modality

In order to obtain increased biomass production and, therefore, higher titer of enterocin, fed-batch fermentations were implemented and scaled up. Two kinds of fed-batch strategies, different for the mode of feeding of fresh medium into bioreactor, were performed and compared in terms of biomass production and bacteriocin activity: pulses feeding and continuous feeding.

3.3.1 Pulses fed-batch experiments

Pulses of fresh broth, containing every nutrient concentrated in order to provide, after the addition into the bioreactor, the initial concentration in the medium, were first carried out on 2 L fermenter.
In “one pulse” experiment, the addition was performed at the end of the exponential phase, when the glucose was completely consumed (5 h), as determined in batch fermentations. In “two pulses” experiment, the additions were respectively done at 5 and 7 h, when the first glucose addition was consumed. Finally, a third pulse was performed at 9 h in three pulses fed-batch.
Figure 6 reports the comparison of the growth curves for these experiments. As expected, increasing the number of pulses, the exponential phase is prolonged and the biomass production is improved. Particularly, in the fermentation with three pulses, a final biomass concentration of 4.3 $g \cdot L^{-1}$, thus improving about three fold the biomass production obtained in batch.
Bacteriocin activity was also improved: the maximum activity, detected after 11 h of fermentation for three pulses experiment, was 160 $BU \cdot mL^{-1}$. Moreover, in terms of specific production, there was not improvement (14.4 $BU \cdot mL^{-1} \cdot OD^{-1}$ against 26.6 $BU \cdot mL^{-1} \cdot OD^{-1}$ obtained in batch fermentation; data not shown).

3.3.2 Continuous feeding experiments and scale up

Fed-batch experiments with continuous feeding of fresh concentrated medium into bioreactor were initially performed on 2 L scale. The feeding strategy was implemented through equations 1 and 2 (see Materials and Methods), using the data (Y_{xs}, μ, q_s) obtained from the analysis of batch fermentations and considering glucose as limiting substrate. In figure 7 the growth curve obtained with this fed-batch strategy is reported

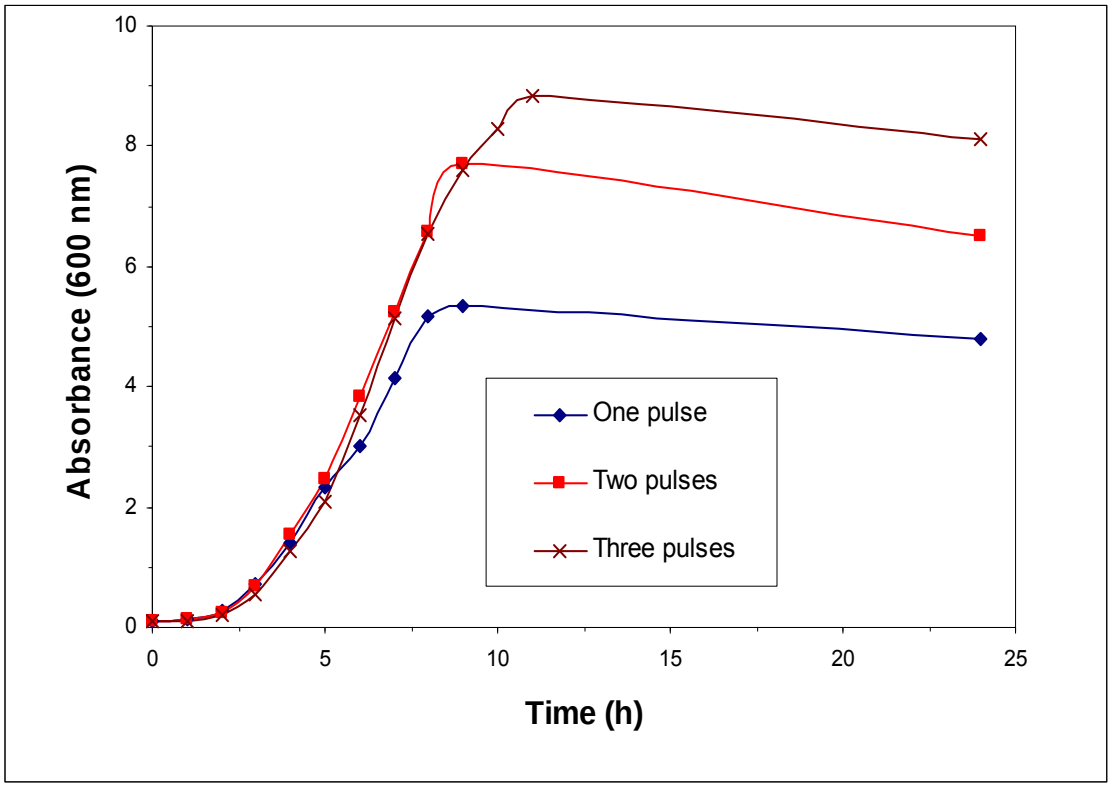

Figure 6
Comparison of the growth curve for *E. casseliflavus* CGA1 in pulses fed-batch fermentation.

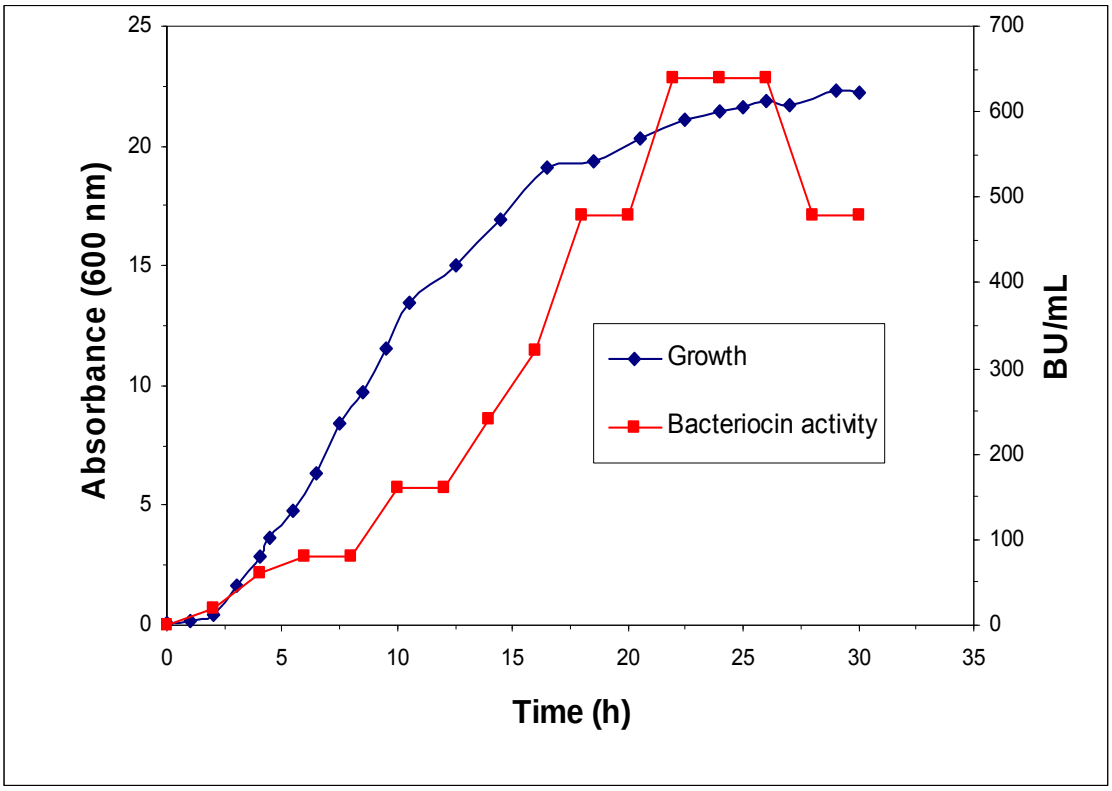

Figure 7
Growth curve of *E. casseliflavus* CGA1 grown through fed-batch fermentation with continuous feeding and kinetics of enterocin production.

and compared to the enterocin activity. The feeding started after 6 h of batch fermentation, when the glucose was completely consumed. The figure clearly shows as the developed feed profile allowed to prolong the exponential phase, thus increasing the biomass production. After 30 h of cultivation, the biomass concentration was 11.6 $g \cdot L^{-1}$. It corresponds to an improvement of 8.5 and 2.5 fold in comparison with batch and pulses fermentations, respectively. The specific growth rate, measured for the fed-batch phase (6-30 h), was 0.07 h^{-1}. The Y_{xs} was kept constant at 0.20 $g \cdot g^{-1}$, similar to the batch fermentations, through the fed-batch phase.
Interestingly, the bacteriocin activity was strongly improved. In figure 7 you can see the peptide was produced as the biomass concentration increased, reaching a maximum activity of 640 $BU \cdot mL^{-1}$ after 22 h of fermentation. Then, the level of product is kept constant, while a decrease to 480 $BU \cdot mL^{-1}$ after 28 h of growth is observed. Considering the maximum bacteriocin concentration, a specific production of 30.4 $BU \cdot mL^{-1} \cdot OD^{-1}$ was obtained.
This process was also scaled up on Biostat C and Biostat D fermenters. The data compared are reported in Table 11, showing a good reproducibility. In fact, very similar values of biomass concentration were obtained; only the cultivation in 100 L fermenter resulted in slightly lower yield, being characterized by a final cell concentration of 10.3 $g \cdot L^{-1}$ against values of 11.6 and 12.5 $g \cdot L^{-1}$ obtained for 2 L and 15 L cultures, respectively. Furthermore, very close values of specific growth rate were obtained, ranging from 0.18 h^{-1} (for 100 L culture) to 0.21 h^{-1} (for 15 L culture).
Very interestingly, the same bacteriocin production was detected, in a time interval from 22 to 24 h.

3.4 Downstream processes

After fed-batch fermentation with continuous feeding on 2 L scale, the broth was processed in purification operations in order to recover the bacteriocin. Downstream processes based on buthanol/acetone, methanol/chloroform extraction or membrane processes were performed, as described in Materials and Methods. In every case, the cells were previously separated by centrifugation and the supernatant was recovered and ready to be further processed.
Table 12 compares bacteriocin activities and yields detected in each fraction for the three downstream processes. The yields for each step are calculated comparing the activity of the step with the initial one in the supernatant (640 $BU \cdot mL^{-1}$). The protein concentrations ($mg \cdot mL^{-1}$) for each step and the increase in specific activity ($BU \cdot mg^{-1}$) are also reported.
Using the buthanol/acetone protocol (Table 12a), a final bacteriocin concentration of 1600 $BU \cdot mL^{-1}$ was obtained from the initial 640 $BU \cdot mL^{-1}$ of the supernatant. Considering the 10 fold concentration factor, this purification gave a yield of enterocin of 25 %. The increase in bacteriocin specific activity resulted in final 2285.7 $BU \cdot mg^{-1}$, that corresponds to a purification fold of 15.
The final yield and purification fold obtained with choloroform/methanol procedure (Table 12b) were 50 % and 11.6, respectively. The chloroform/methanol contained 3200 $BU \cdot mL^{-1}$ of bacteriocin and 1.8 $mg \cdot ml^{-1}$ of residual proteins, corresponding to a specific activity of about 1780 $BU \cdot mg^{-1}$.
For the membranes purification protocol, experiments to optimize the process

Table 11
Comparison of fed-batch fermentations on 2, 15 and 100.

Culture volume (Fermenter)	Final X ($g \cdot L^{-1}$)	μ (h^{-1})	Maximum $BU \cdot mL^{-1}$ (h)	Maximum $BU \cdot mL^{-1} \cdot OD^{-1}$ (h)
2 L (Biostat CT)	11.6	0.19	640 (22)	30.4
15 L (Biostat C)	12.5	0.21	640 (24)	31.2
100 L (Biostat D)	10.3	0.18	640 (22)	31.0

Data of bacteriocin activity are in triplicates with standard deviation included between 5-10 %.

Table 12
Comparison of the downstream processes of the enterocin from fed-batch fermentation broth.

(a)

Parameter	Supernatant	Buthanol extract	Acetone precipitate
BU·mL^{-1}	640	3200	1600
mg·mL^{1}	4.9	2.2	0.7
BU·mg^{-1}	152.3	1454.5	2285.7
Purification fold	0	9.5	15
Yield (%)	100	50	25

(b)

Parameter	Supernatant	Ammonium sulphate precipitate	Chloroform/methanol extract
BU·mL^{-1}	640	4800	3200
mg·mL^{1}	4.9	3.2	1.8
BU·mg^{-1}	152.3	1500	1777.7
Purification fold	0	9.8	11.6
Yield (%)	100	75	50

(c)

Parameter	Supernatant	Permeate (30 kDa)	Ammonium sulphate precipitate	Retentate (3 kDa)
BU·mL^{-1}	640	480	4800	9600
mg·mL^{1}	4.9	3.6	2.1	1.1
BU·mg^{-1}	152.3	133.3	2285.7	8727.2
Purification fold	0	0.8	15	57.2
Yield (%)	100	75	75	75
Purification fold - 15 L	0	0.6	11.2	51.2
Yield - 15 L (%)	100	75	75	75

Data of bacteriocin activity are in triplicates with standard deviation included between 5-10 %.

parameters of the ultrafiltration step at 3 kDa were initially performed. These experiments were finalized to evaluate the best cross flow rate and TMP to perform the process, that are the conditions that maximize the permeate flux.
Figure 8 reports, as result of these experiments, the plot of permeate flux versus TMP at the different retentate flows tested. The best conditions are the retentate flow and the TMP that maximize the flux, before the levelling of the curve: retentate flow of 200 $mL \cdot min^{-1}$, corresponding to a cross flow rate of 14.2 $lpm \cdot m^{-2}$, and TMP of 1.25 bar.
In Table 12c the results of set of experiments are summarized. The yield of the first step (30 kDa ultrafiltration) was 75 %. However the remaining steps did not cause further decrease of the product; in fact, no other losses were detected in the ammonium sulphate precipitation and 3 kDa diafiltration. Finally 9600 $BU \cdot mL^{-1}$ of enterocin were detected in the 3 kDa retentate, with a 20 fold concentration factor. In terms of specific activity, no improvement was obtained by the 30 kDa ultrafiltration (about 133 $BU \cdot mg^{-1}$), while a great increase was obtained in the next steps: 2285.7 $BU \cdot mg^{-1}$ for ammonium sulphate precipitation and 8727.2 $BU \cdot mg^{-1}$ for 3 kDa ultrafiltration. This corresponded to a final purification fold of over 57.
The downstream processing based on membranes was also scaled up to a larger volume. In particular, the broth from Biostat C (15 L fermenter) was recovered and processed through this procedure. Table 12c reports the purification folds and the yields obtained. Despite of a lower specific activity of the ammonium sulphate step than the small scale precipitation, the 3 kDa ultrafiltration allowed to deeply purify the enterocin until to have a final purification of about 51 fold, very close to the value obtained in the 2 L procedure (57.2). Both the recovery for each step and the final yield were the same (75 %).

3.5 Determination of the enterocin molecular weight

Ultrafiltration experiments on centrifugal filters with different cut offs were performed in order to identify the range of enterocin molecular weight. Supernatant of fed-batch fermentation was used at this aim (initial bacteriocin activity of 640 $BU \cdot mL^{-1}$). The activity of the fraction over 10 kDa was 160 $BU \cdot mL^{-1}$ (data not shown). A greater concentration (480 $BU \cdot mL^{-1}$) was detected in the fraction between 3 and 10 kDa. No activity was observed below 3 kDa.
A further evidence that the molecular weight of the product ranges between 3 and 10 kDa was obtained by tricine SDS-PAGE. Figure 9 shows the electrophoretic gel for the supernatant after batch fermentation, fed-batch fermentation with continuous feeding and purification through ultrafiltration steps. The picture shows as, after performing the downstream processing of the fermentation broth through membranes, only one protein band, corresponding to the enterocin (as demonstrated by the activity assay), is obtained. The molecular weight of the product, as showed by the electrophoretic gel, ranges between 4.5-5 kDa.

3.6 Activity spectra

The fermentation supernatants and the purified enterocin were tested against a number of Gram positive and Gram negative bacteria, using the agar well diffusion assay. The results are showed in Table 13. It can be seen that the enterocin has activity versus 7

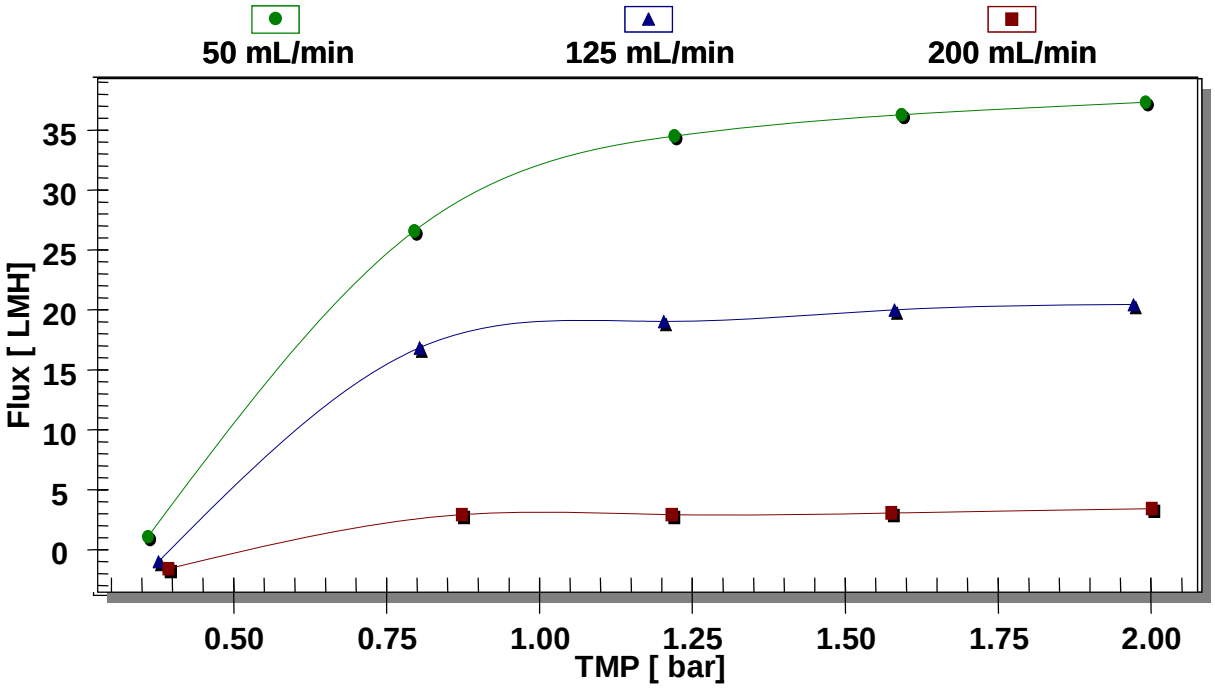

Figure 8
Optimization curves for 3 kDa ultrafiltration process at three different retentate flows. 3 kDa hollow fibers were used (filtering area=0.014 m^2).

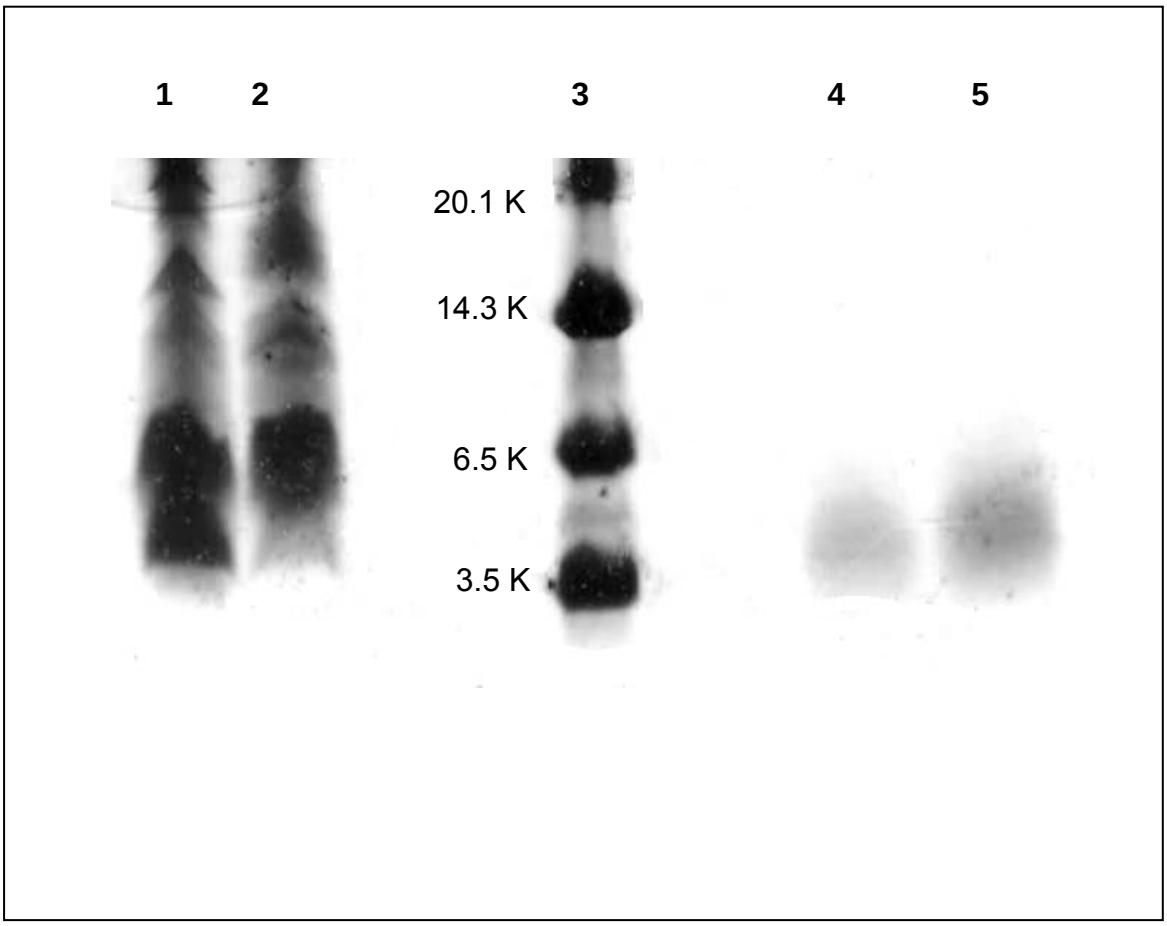

Figure 9
Tricine SDS-PAGE of supernatant of the fed-batch fermentation (lane 1), supernatant of the batch fermentation (lane 2), product after membranes purification protocol (lane 4) and 2 fold concentrated product after membranes purification protocol (lane 5). Lane 3: molecular weight markers.

Table 13
Spectrum activity of the bacteriocin produced by *E. casseliflavus* CGA1, *L. plantarum* DSM 12028 and *L. crispatus* L1 towards some Gram positive and negative bacteria.

Microorganism target	Gram	Properties	*E. casseliflavus* CGA 1	*L. plantarum* DSM 12028	*L. crispatus* L1
Lactobacillus casei DSM 20011	+	Dairy starter	++	-	-
Lactobacillus bulgaricus DSM 21081	+	Dairy starter	+	-	-
Lactobacillus crispatus L1	+	Vaginal isolated with probiotic function	+++	-	//
Lactobacillus plantarum DSM 12028	+	Meat starter	-	//	-
Lactococcus lactis NZ9000	+	Dairy starter	++	+	-
Listeria monocytogenes	+	Human pathogen	+++	-	-
Staphilococcus aureus	+	Human pathogen	+	+++	+++
Streptococcus agalactiae	+	Human pathogen	-	++	++
Enterococcus faecium	+	Human pathogen	+++	-	-
Klebsiella pneumoniae	+	Human pathogen	-	+	++
Escherichia coli K4	-	Human pathogen	-	+	++
Escherichia coli BL21	-	Human pathogen	-	++	+

-= Absence of inhibition; + = Inhibition halo between 8 and12 mm; + + = Inhibition halo betwwen 12 and 16 mm; + + + = Inhibition halo > 16 mm

out 12 tested microroganisms. Particularly, a great activity (inhibition halo over 16 mm) was detected versus strains of *L. monocytogenes, Enterococcus faecium* and *Lactobacillus crispatus* L1, while less inhibitory ability was observed versus the other microrganisms (inhibition halo under 16 mm). Besides *L. monocytogenes*, only *Staphilococcus aureus* was weakly inhibited, between pathogens tested. No inhibition was detected against *Klebsiella pneumoniae*, *Streptococcus agalactiae* and *Escherichia coli* strains tested. The probiotic strain *L. plantarum* DSMZ12028 was also not inhibited by the enterocin. An agar plate that shows the inhibition towards *L. monocytogenes* is in figure 10.

The table also shows the inhibitory spectrum of *Lactobacillus plantarum* DSM 12028 and *Lactobacillus crispatus* L1. The first one was able to inhibit *S. aureus* (inhibition halo over 16 mm), *S. agalactiae* and *E. coli BL21*, while less activity was observed against *K. pneumoniae*, *E. coli* k4 and *L. lactis* NZ9000. *L. monocytogenes* and the other starter/probiotic strains tested were not inhibited. A similar activity spectrum was defined for *L. crispatus* L1. Particularly, a strong activity was detected versus *S. aureus* (inhibition halo over 16 mm). The pathogens *S. agalactiae*, *K. pneumoniae*, *E. coli* K4 and *E. coli* BL21 were also inhibited.

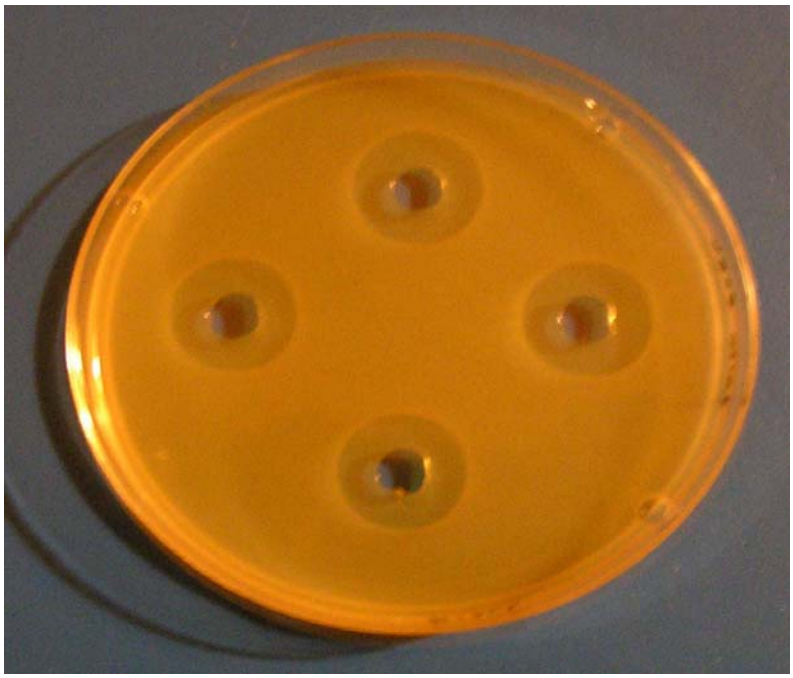

Figure 10
Agar well diffusion assay of the enterocin produced by *E. casseliflavus* CGA1 towards with *L. monocytogenes*. Clear halos indicate zones where the microorganism was inhibited.

CHAPTER IV

4. DISCUSSION

Lactic acid bacteria (LAB) are exstensively used in food processing, such as in dairy and meat fermented products for their contribution to shelf life, texture, and organoleptic functions (Wood B.J.B., 1997). Due to these properties, most of them are generally regarded as safe (GRAS) in food production. These microorganisms have furthermore been used in food and feed preservation for centuries since they can produce a variety of antimicrobial agents, including organic acids like lactic and acetic acid, ethanol, carbon dioxide, diacetyl, and hydrogen peroxide (Holzapfel et al., 1995). Additionally, many LAB produce a high diversity of different bacteriocins (Cleveland et al., 2001).
Bacteriocins are antibacterial peptides or proteins produced by bacteria that kill or inhibit the growth of other microorganisms, particularly strains closely related to the producer one. Most of them are found in numerous fermented and non-fermented foods, e.g. meat, fish, dairy products and vegetables. Furthermore, some of them inhibit spoilage bacteria and food-borne pathogens including *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum*. For this reason, the use of either bacteriocins produced by LAB strains as food preservative or the bacteriocin-producing LAB starter cultures has received a special attention as a new preservation method to control pathogenic bacteria, based on a completely “natural” approach. The number of research papers on this topic is increasing all over the world. However, though many bacteriocins have been biochemically and genetically characterized and there is basic understanding of their structure-function, biosynthesis, and mode of action, though many factors influencing their production in food systems are clarified, nisin, a lantibiotic produced by *Lactococcus lactis* ssp. fermentation, is currently the only bacteriocin actually used as food preservative. The reason for this resides primarily in the lack of suitable and economically convenient processes for bacteriocin production on industrial scale, in terms both of fermentative processes and adequate purification procedures. Indeed, for effective commercial application, production processes have to be optimized, by establishment of the optimal conditions for growth and bacteriocin production and recovery.
The aim of the present work was to study the production of bacteriocins from three LAB stains (*Enterococcus casseliflavus* CGA1, *Lactobacillus plantarum* DSM 12028 and *Lactobacillus crispatus* L1) assaying their ability to inhibit pathogenic microorganisms and developing productive processes scalable for an industrial application. Particularly, for one of these strains, belonging to *Enterococcus* genus and obtained by an industry active in food processing, a fermentative process aiming to bacteriocin production was optimized and scaled up to a pilot plant. Also the downstream processing to purify the product from fermentation broth was developed.
Among LAB genera, enterococci frequently occur in some cheeses and meats, particularly in those produced in Mediterranean countries (Sarantinopoulos et al., 2002). They are considered as either a natural starter culture or a contamination from the environment, contributing to the development of the organoleptic properties of the food. Very interestingly, bacteriocin-producing enterococci are widespread in nature and have been isolated from numerous sources (Giraffa G., 1995), such as dairy products, fermented sausages, fish, vegetables, and the mammalian gastrointestinal tract. Several enterococcal bacteriocins (enterocins) have been characterized at the molecular level and identified as generally belonging to class II and having the potential to inhibit the

growth of a narrow range of pathogenic strains, above all *Listeria monocytogenes* ssp. (Sabia et al., 2003). For this reason, bacteriocins produced by enterococci might be good candidates for potential application of bacteriocin-mediated antagonism against *L. monocytogenes* in foods.

Enterococcus casseliflavus CGA1, purchased by the industry to our research center, has been isolated by "salami" meat and delivered to characterize its ability to produce an enterocin active towards *Listeria monocytogenes*. In attempt to develop a productive process for this bacteriocin scalable on an industrial level, first the medium for microorganism growth and enterocin production was optimized. It's widely reported in literature that bacteriocin production from LAB is strongly influenced by several factors, mainly cultural conditions, such as pH, temperature and medium composition (Parente and Ricciardi, 1999).

As far as the medium composition is concerned, rich and complex media as MRS give highest yields of bacteriocin. For example, high bacteriocin levels were obtained by *Lactobacillus plantarum* strains ST23LD and ST341LD grown in MRS medium (Todorov and Dicks, 2006). Futhermore, the addition of complex sources, as tryptone, yeast extract and meat extract, could significantly improve the bacteriocin production. Similarly the addition of yeast extract to a medium based on milk improved the bacteriocin production by some LAB strains (Avonts et al., 2004). High bacteriocin levels were obtained from a number of LAB strains grown in media based on MRS (Leroy and De Vuyst, 2002; Leal-Sánchez et al., 2002). Despite the high bacteriocin production, these media are expensive and, therefore, not suitable for industrial applications. In order to evaluate the effect of medium composition on enterocin production from *E. casseliflavus* CGA1, flask experiments were performed. The microrganism was grown in MRS and in a basal medium, whose composition was suggested by our industrial partner (Table 5), added with different industrial complex sources. Being the bacteriocin a peptide, the use of nitrogen nutrients with different compositions and concentrations of several amino acids and low molecular weight peptides could have influence on the bacteriocin production, besides the microorganism growth. Indeed, the growth and the bacteriocin production in presence of some sources, as soy peptones and yeast extract, was better than in presence of other ones, as malt extract. Particularly, a bacteriocin activity of 80 $BU \cdot mL^{-1}$ was obtained with the two soy peptones tried. In terms of bacteriocin specific production, that is the bacteriocin activity produced per biomass unit, the best result was 20.5 $BU \cdot mL^{-1} \cdot OD^{-1}$, obtained when the medium was added with soy peptone A2SC. These results demonstrate that the medium composition, and mainly the kind of complex nitrogen source, has effect on the bacteriocin production from LAB. The absence of bacteriocin activity detected in the medium added with malt extract may be explained considering that this nutrient is primarily constituted by carbohydrates, rather than amino acids and peptides. As expected, a good result was also obtained in the growth experiment with MRS. After 8 h of growth, also 80 $BU \cdot mL^{-1}$ were detected, but the specific bacteriocin production was better (22.7 $BU \cdot mL^{-1} \cdot OD^{-1}$) than the growth in soy peptone A2SC. As anticipated before, the drawbacks of using rich media as MRS for an industrial implementation reside in a high cost, and in the complexity of their composition, with consequent problems related to easy growth of other microorganisms and to eventual contamination in the purification procedure. On the contrary, the medium supplied with soy peptone A2SC is a cheaper medium at known composition, able to give good enterocin production by *E. casseliflavus* CGA1.

In order to further reduce the cost of the medium, other flask experiments were carried out. Different combinations of glucose (carbon and energy source) and soy peptone A2SC concentrations from 20 to 5 $g \cdot L^{-1}$ were assayed. As expected, an influence on biomass production was observed, but it mainly depended on the soy peptone A2SC, further demonstrating the key importance of the complex source for the growth of fastidious microorganisms as LAB. Particularly, the reduction of this nutrient from 20 to 10 $g \cdot L^{-1}$ did not influence greatly the biomass production, while the further decrease to 5 $g \cdot L^{-1}$ has effect: at 8 h (last point of exponential phase), the OD value is about 2.8 every time that soy peptone A2SC concentration is 5 $g \cdot L^{-1}$**,** independently if glucose concentration is 5, 10 or 20 $g \cdot L^{-1}$. Only at 24 h, a reduction of biomass to an OD value of 2.5 is observed in the medium with 5 $g \cdot L^{-1}$ for both glucose and soy peptone A2SC, indicating that the absence of the carbon and energy source for maintenance reactions can cause the starting of cell lysis in late stationary phase. Of course, the depletion of nitrogen source has influence on bacteriocin production, that goes down to 60 $BU \cdot mL^{-1}$ when soy peptone concentration is 5 $g \cdot L^{-1}$ against the initial 80 $BU \cdot mL^{-1}$. This effect can be explained considering the proteinaceous nature of the enterocin and the lower biomass production. Interestingly, the bacteriocin production per biomass unit is greater in the media containing low concentrations of soy peptone A2SC and glucose (22.9 $BU \cdot mL^{-1} \cdot OD^{-1}$), and very close to the specific production obtained in MRS. The reduction of glucose and complex source from 20 to 5 $g \cdot L^{-1}$ has two main beneficial effects on the development of the process: on one side, a significant drop of the fermentation medium cost is obtained; on the other one, the downstream processing is facilitated because less impurities have to be removed, above all considering that soy peptone is a source of peptides, potential contaminants of the product.

The next step in process development was the study of the microorganism growth and enterocin production in fermenters operating in batch modality. Batch fermentations have been often used to understand the correlation between bacteriocin production and cultural conditions, e.g. pH and temperature, and to characterize the kinetics of bacteriocin production. For example, Parente and co-workers (1997) demonstrated how the bacteriocin production by *Enterococcus faecium* DPC1146 is strictly dependent on the growth phase, starting as soon as the cell growth begins and ceasing immediately at the end of exponential phase. A similar trend of production was observed for most of the bacteriocins produced by LAB grown in bach modality. Also the production of enterocin from *E. casseliflavus* CGA1 follows a typical kinetics of primary metabolite. The peptide production starts in concomitance with the starting of the exponential phase, thus reaching a maximum of 80 $BU \cdot mL^{-1}$ at the end of it. This value is kept constant during the stationary phase. At 24 h, a decrease of activity to 60 $BU \cdot mL^{-1}$ is observed. This drop is very often observed for bacteriocin production and can be explained as a result of bacteriocin degradation by specific or non specific proteases (De Vuyst and Vandamme, 1992), protein aggregation with consequent loss of activity and pH-dependent adsorption of the bacteriocin molecules to the membrane of the producer cells (Yang et al., 1992). The optimal pH for *E. casseliflavus* CGA1 is 7; and it has been observed that the adsorption of bacteriocins to cells is maximal at neutral pH and decreases at low pH. On the other hand, no reduction of bacteriocin titre was observed when fermentations were carried out without pH control (Yang and Ray, 1994). In comparison with flask experiments, the biomass production was the same but the exponential phase ended earlier (higher specific growth rate) and a slight improvement

of bacteriocin activity was detected, as expected growing the microorganism in a more controlled environment.
Besides the characterization of bacteriocin production, batch fermentations allowed to evaluate the profile of biomass and lactic acid production on glucose. Biomass yield of on glucose (Y_{xs}) was 0.22 $g \cdot g^{-1}$, typical for a microorganism with fermentative metabolism, while the yield of lactic acid on glucose (Y_{ps}) was 0.89 $g \cdot g^{-1}$, demonstrating the homolactic character of the microorganism when grown on glucose. In fact, Y_{ps} of 0.70-0.95 $g \cdot g^{-1}$ are tipically observed when LAB degrade glucose through homolactic fermentation, producing lactic acid as main by-product.
A crucial step in the optimization of a bioprocess is represented by the implementation on higher scale, this is needed to support the feasibility of an industrial process. The three main scales for bioprocess development are laboratory (1-100 L), pilot plant (100-1000 L) and production (more than 1000 L), but some authors include in this classification also the shake flask scale (Votruba and Sobotka, 1992). For biotechnological processes, the scale up ratio is typically about 1:10 up to 100000 L but lower ratios of about 1:5 often have been used to increase comfort levels (i.e., decreased risk of unexpected performance on scale up). The starting point to scale up a biotechnological process is the maintenance of identical operating conditions (usually assuming that there is geometrical similarity). Batch fermentations of *E. casseliflavus* CGA1 on the optimized medium were performed on three different volumes (2, 15 and 100 L), using a scale up ratio of 1:10 and maintaining the same operating conditions and bioreactor configuration. Very interestingly, this linear scale up strategy allowed to obtain a good reproducibility of the data, in terms of bacterial growth (biomass production and specific growth rate) and yields on glucose (Y_{xs} and Y_{ps}). Very similar bacteriocin production kinetics, with a peak at the end of exponential phase and a decrease in late stationary phase, was also detected. Therefore, no need to change operating parameters or reduce the scale up ratio was taken in consideration.
In attempt to obtain higher enterocin productions, fed-batch strategies were implemented. Differently by batch fermentations, where the bacteriocin production ceases at the end of growth phase and the yield is usually low, fed-batch fermentation technology allows to obtain high cell densities through the supply of fresh medium and, therefore, as bacteriocins are growth-related metabolites, higher bacteriocin titer (Callewaert and De Vuyst, 2000). In particular, an improvement of biomass production of *E. casseliflavus* CGA1 was observed in pulses fed-batch experiments: as expected, increasing the number of pulses, the final biomass concentration also increases, reaching 4.3 $g \cdot L^{-1}$ in the experiment with three additions, that corresponds to a three fold improvement in comparison with the biomass production obtained in batch. Interestingly, a lower bacteriocin specific production (14.4 $BU \cdot mL^{-1} \cdot OD^{-1}$ against 26.6 $BU \cdot mL^{-1} \cdot OD^{-1}$ obtained in batch fermentation) was detected, indicating that the increase in biomass does not corresponds to a proportional increase in bacteriocin amount.
In order to improve the bacteriocin specific production through fed-batch fermentations, a different strategy of medium addition, based on continuous feeding into bioreactor, was developed using the data of yield and glucose consumption obtained by the analysis of batch fermentations. A real increase in biomass production was obtained, improving batch and pulses fed-batch fermentation of about 8 and 2 fold, respectively. The difference in feeding modality has also influence on bacteriocin production. After 22 h of fermentation 640 $BU \cdot mL^{-1}$ are produced, corresponding to a 8 fold improvement of

the batch activity and a bacteriocin specific production of 30.4 $BU \cdot mL^{-1} \cdot OD^{-1}$. Even tough the culture slightly continues to grow, a decrease of bacteriocin activity after 28 h of growth is observed, probably due to pH-dependant adsorption phenomena to the producer cell membrane.

Fed-batch technology based on continuous feeding are already used to improve the production for some bacteriocins. For example the production of amylovorin L471 by *Lactobacillus amylovorus* DCE 471 was improved in fed-batch fermentation if substrate addition is controlled by acidification rate of the culture (Callewaert and De Vuyst, 2000). In a similar study, a fed–batch strategy based on exponential feeding was used to improve the bacteriocin production by *Lactobacillus curvatus* CWBI-B28 (Ghalfi et al., 2007). Despite the increase of the bacteriocin levels, the drawback of these fed-batch processes is the use of very rich media, both for batch (MRS containing addition of glucose and of different complex nitrogen sources) and fed-batch phase (high concentrations of glucose and complex nitrogen sources).

The robustness of the developed fed-batch strategy was confirmed in linear scale up experiments until pilot plant of 100 L. Very interestingly, the same maximum bacteriocin activity was obtained (640 $BU \cdot mL^{-1}$) in a time interval between 22 and 24 h. These experiments demonstrate the suitability of the developed process for the implementation on an industrial scale.

After fermentation, the product has to be recovered in order to have high purity and yield. The purification phase represents very often the most expensive step of the whole production process. The optimization of the downstream procedure is therefore essential to develop an applicable and cost effective process. Very few processes have been developed to purify bacteriocins from fermentation broths. Generally, bacteriocins are purified by subsequent centrifugation, ammonium sulphate precipitation and one or more chromatographic steps (Carolissen-Mackay et al., 1997). Although these procedure function reasonably well when small quantities of peptide have to be purified from a small (<1-liter) bacterial culture, they tend to become unmanageable for the large volumes needed for industrial production. In order to overcome this problem, downstream processes based on utilization of large amounts of organic solvents for precipitation and extraction operations, as buthanol, acetone or chloroform, have been developed. Although solvents may avert most of impurities from the broth, they are very expensive and also legal and security constraints should be considered for the bacteriocin purification from fermentation broths. These drawbacks make these procedures not suitable for industrial scale applications.

In order to avoid the use of organic solvents, our objective was to design and develop an innovative downstream process based on the tangential flow filtration (TFF) technology.

This technology is increasingly being exploited in recovery schemes for biotechnological products. Its applications range from bacterial and mammalian cells harvesting and virus removal to concentration of proteins and nucleic acids and buffer exchange applications (van Reis and Zidney, 2001). Processes based on membranes take advantage of their selectivity, high capacity and very low environmental impact. Furthermore, they are very well suited to the processing of biological molecules since they operate at relatively low temperatures and pressures and involve no phase changes or chemical additives, thereby minimizing the extent of denaturation, deactivation, and/or degradation of biological products. Finally, TFF cartridges may be used in numerous cycles and linearly scalable devices are commonly manufactured.

The downstream processing based on membranes we developed consisted of two ultrafiltration steps. The first one is a purification on 30 kDa membranes of the cell-free fermentation broth by large impurities, as cell debris or proteases active towards the peptide. The enterocin, having a low molecular weight, passes through the membrane and is recovered in the permeate. After an ammonium sulphate precipitation, a concentration and diafiltration step to remove the salts and low molecular weight impurities and obtain the peptide in purified water is performed on 3 kDa membranes. This last process was properly optimized in terms of TMP and cross flow rate that maximize the permeate flux. A permeate flux of 35 LMH was obtained at not drastic conditions of TMP and recirculation flow rate. Although the cut-off of the membrane is small, this reasonably high flux can be explained considering the relatively high purity of the product at this point of the downstream processing.
The developed purification procedure was compared with a consolidated protocol of extraction/purification based on buthanol/acetone and a procedure based on chloroform/methanol reported in literature (Callewaert et al., 1999; Foulquié Moreno et al., 2003). Using the buthanol/acetone strategy, only the 25 % of the product was recovered, with a final purification fold of 15. Both buthanol extraction and acetone prepicipation were characterized by product losses. A better enterocin yield (50 %) was obtained with the chloroform/methanol procedure, despite of a low purification factor (11.6), indicating as this strategy is not selective for the product of interest. The resuts obtained show as these procedures are not suitable for a real and cost effective industrial application. On the contrary, when the procedure based on membranes was used to recover the enterocin, a final yield of product of 75 % was achieved. Furthermore, a greater final concentration factor (20 fold) and a relatively high purification fold of 57.2 were obtained, in comparison with procedures based on organic solvents. The purity of the product after this purification was clearly showed by tricine SDS-PAGE: only one protein band is obtained at a molecular weight of 4.5-5 kDa.
Losses of product were observed in the 30 kDa ultrafiltration step: although the bacteriocin has a low molecular weight, part of product molecules pool is retained in the concentrate. This retention can be explained considering the following phenomena: aggregation of the peptide molecules forming high molecular weight polymers and interactions between the peptide and/or the aggregates and the membrane material at the surface or wiithin the pores. Bhugaloo-Vial and co-workers (1997) observed retention of divercin on 60-80 kDa membranes in a bioreactor coupled to the microfitration. They explained this behaviour hypotizing the formation of large complexes of bacteriocin in the fermentation conditions. Also helveticin J was produced as a complex superior to 300 kDa in culture supernatants while the purified product was a protein of 37 kDa (Joerger and Klaenhammer, 1986). Acidocin B activity was found at approximately 100 kDa when purified through gel permeation (Barefoot and Klaenhammer, 1984). The high apparent molecular weight of crude acidocin B resulted from the occurrence of large aggregates in the fermented MRS broth.
Interestingly, no losses were detected in the next ammonium sulphate precipitation, differently from the chloroform/methanol procedure, where losses of product of 25 % occurred. That could be explained considering that the preceding ultrafiltration step removes impurities that interfere with the precipitation, e.g. aggregates between bacteriocins and/or bacteriocins and cell components that do not precipitate.

The downstream processing based on membranes was also scaled up on the 15 L scale. The final yield was the same. In terms of purification from proteins, only the ammonium sulphate precipitation gave a worse result, while the two ultrafiltration steps were perfectly reproduced. These results show the potentiality of the developed procedure to be transferred on an industrial scale, expecting the same performance obtained on laboratory scale.

The purified product was used to characterize the activity spectrum of *E. casseliflavus* CGA1 versus pathogenic and beneficial microorganism. Very interestingly, strong activity versus *Listeria monocytogenes* was detected. Between the non-pathogenic strains, only *Lactobacillus plantarum* DSM 12028, a meat starter isolated by "salami", was not inhibited. These two results are very comforting for the final objective of the project, that is the application of the enterocin as meat preservative to control the contamination by pathogens as *L. monocytogenes*. Of course, it is critical that the product does not have inhibitory activity towards beneficial and naturally occurring microorganisms of the food, as *L. plantarum* strains.

The same microorganisms tested for defining the spectrum activity of *E.casseliflavus* CGA1 were used to assay the inhibitory activity of two LAB belonging to *Lactobacillus* genus: *Lactobacillus plantarum* DSM 12028 and *Lactobacillus crispatus* L1. The first one, isolated by meat, as above mentioned, was able to strongly inhibit *Staphilococcus aureus*, typical food pathogen, and, at a lower extent, other pathogens, as *Streptococcus agalactie* and *Escherichia coli* BL21. The same activity towards *S. aureus* was detected for *L. crispatus* L1, vaginal isolated with probiotic function. *S. agalactiae*, *Klebsiella pneumoniae* and *E.coli* K4 were also inhibited at a good extent. No activity towards *L. monocytogenes* was observed for the two strains. Differently from *E. casseliflavus* CGA1, the lactobacilli were able to inhibit Gram negative *E. coli* strains, in presence of EDTA. This chelating agent is able to sequestrate bivalent ions, e.g. magnesium, from lypopolysaccharidic layer of Gram negative bacteria outer membrane, destabilizaing it and allowing the antimicrobial peptide to carry out its action (Haque and Russel, 1975). For their action towards different pathogenic microorganisms, bacteriocin produced by *L. plantarum* DSM 12028 and *L. crispatus* L1 seem to have promising application in the field of biological preservation of foods.

Therefore, flask experiments were performed to study the effect of different concentrations of bactocasitone (complex nitrogen source tipically used in media to cultivate lactobacilli, Kimmel and Roberts, 1998). For both microorganisms, no bacteriocin activity was detected when the concentration of bactocasitone was 1 $g \cdot L^{-1}$**,** indicating as high amounts of complex nitrogen source are required to have a basal production of bacteriocin. 5 $g \cdot L^{-1}$ are sufficient for *L. plantarum* DSM 12028 to have 40 $BU \cdot mL^{-1}$ while 10 $g \cdot L^{-1}$ are required for *L. crispatus* L1 to have the same bacteriocin activity. The latter case is a demonstration that conditions that improve growth also support the bacteriocin production.

Batch experiments were performed in order to evaluate the effect of controlled growth conditions on bacteriocin production from the two *Lactobacillus* strains. Improving the biomass production, also the bacteriocin activity was significantly improved, particularly for *L. plantarum* DSM 12028. In this case, the bacteriocin activity was improved three fold, along with specific production (14.4 $BU \cdot mL^{-1} \cdot OD^{-1}$). On the contrary, a slight decrease of specific production in comparison with flask experiments was detected for *L. crispatus* L1. The data obtained, particularly for *L. plantarum* DSM12028, confirm the

impression that this microorganism could be a good candidate as bacteriocin-producing strain, potentially applicable in food preservation.

CONCLUSIONS

Three LAB strains were screened to evaluate their ability to produce bcateriocins potentially interesting in food industry. For one of them (*Enterococcus casseliflavus* CGA1), able to produce a peptide active towards *Listeria monocytogenes*, a biotechnological process for production and recovery of the molecule, based on modern fermentation and purification technologies, was developed. The research work has addressed towards different aspects of the process optimization. First, studies on flask scale allowed to identify the composition of a suitable medium for microorganism growth and bacteriocin production, then batch and fed-batch fermentation techniques have been exploited to enhance the production and finally scale up studies have been carried out until a pilot plant fermenter. Contemporary, in order to find a suitable purification process, an innovative downstream procedure based on utilization of ultrafiltration technology was developed and compared with traditional strategies. All the experiments indicated that the biotechnological production of the enterocin may be possible. Other experiments need to be performed to test the effective inhibitory action of the purified product in the food system.
In addition, two lactobacilli strains, *Lactobacillus plantarum* CGA1 and *Lactobacillus crispatus* L1, were proved able to produce antimicrobial agents towards some pathogenic microorganisms. Bacteriocin production from these strains was studied through flask and batch experiments, demonstrating their potentiality as producers of interesting bacteriocins for food industry.

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APPENDIX

Conference and course attendance:

- Participation as speaker in "ÄKTAcrossflow User Day" - GE Healthcare, Munich, Germany, October 23-24, 2007 – Presentation title: "Development and optimization of a filtration process using ÄKTA crossflow and scale up to UniFlux 10"

- "Microbial hyaluronic acid production"
Marzaioli I., Vinciguerra S., **D'Avino A**., Andreozzi L., Monteasi R., De Rosa M., Schiraldi C.
LII SIB National Congress – Società Italiana di Biochimica, Riccione, Italy, September 26 - 28, 2007
URL: http://www.biomedia.net/sib2007/

- "Efficient production of a novel antilisterial bacteriocin through fed-batch fermentation on industrial isolated *Enterococcus casseliflavus/gallinarum* CGA1"
D'Avino A., De Rosa M., Donnarumma G., Schiraldi C.
VII SIM Annual Meeting and Exhibition – Society for Industrial Microbiology, Denver, Colorado, USA, July 29 - August 2, 2007
URL: http://www.simhq.org/meetings/07annual/index.html

- Participation in the course "Scale-up, Design and Optimization of Tangential Flow Filtration Systems" - Millipore, Molsheim, France, October 17-18, 2006
URL: http://www.millipore.com/bioprocesstraining

- "*Saccharomyces pastorianus* as cell factory to improve production of metabolites of industrial interest using novel fermentation strategies"
Ruggiero A., **D'Avino A.**, Restaino O., Schiraldi C., De Rosa M.
III International Congress on Biocatalysis – Biocat 2006, Hamburg University of Technology, Hamburg, Germany, September 3-7, 2005
URL: http://www.biocat2006.de/cms/

- Participation as speaker in "Äkta User Club" - GE Healthcare, Wien, Austria, May 11, 2006 – Presentation title: "Practical aspects performing downstream process development with ÄKTA crossflow"
URL:
http://www6.gelifesciences.com/aptrix/upp00919.nsf/Content/Labsep_AKTA~USERClub

- "Optimization of production process of fructose 1,6-diphosphate by permeabilized yeast cells"
D'Avino A., Ruggiero A., De Rosa M, Schiraldi C.
VIII National Congress on Biotechnology, University of Studies of Siena, Siena, Italy, September 7-9, 2005
URL:http://www.unisi.it/eventi/CNB8/inglese/index.htm