ANA ANDRÉA TEIXEIRA DE CARVALHO

Fatores que interferem na produção de bovicina HC5 e obtenção de mutantes não produtores de bacteriocina

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção de título de *Doctor Scientiae*.

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APROVADA: 05 de Junho de 2009

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"Glória seja dada a Deus, que pelo seu grandioso poder operando em nós é capaz de fazer muito mais do que jamais ousaríamos pedir ou mesmo imaginar, infinitamente além de nossas mais sublimes orações, anseios, pensamentos ou esperanças". (Ef 3.20)

"Dele, por Ele e para Ele são todas as coisas. A Deus a glória por toda eternidade!"(Rm 11.36)

Ao meu amado esposo e à minha querida mãe,

dedico.

Ao professor Hilário,

minha eterna gratidão, admiração e respeito.

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BIOGRAFIA

ANA ANDRÉA TEIXEIRA DE CARVALHO, filha de Jésus Teixeira de Carvalho e Maria Teixeira Gomes, nasceu em Viçosa, Estado de Minas Gerais, em 10 de maio de 1977.

Em Março de 1999, iniciou o curso de Economia Doméstica pelo Departamento de Economia Doméstica da Universidade Federal de Viçosa, em Viçosa/MG, onde foi bolsista de iniciação científica pela FAPEMIG. Graduou-se em Janeiro de 2004, Licenciada e Bacharel em Economia Doméstica. Nesse mesmo ano, no mês de Agosto, iniciou o curso de Mestrado em Microbiologia Agrícola no Departamento de Microbiologia da Universidade Federal de Viçosa.

Em 19 de abril de 2006, obteve o título de *Magister Scientiae* em Microbiologia Agrícola. Em maio deste mesmo ano iniciou o curso de Doutorado em Microbiologia Agrícola nesta mesma universidade.

SUMÁRIO

RESUMO

CARVALHO, Ana Andréa Teixeira de, D.Sc. Universidade Federal de Viçosa, junho de 2009. **Fatores que interferem na produção de bovicina HC5 e obtenção de mutantes não produtores de bacteriocina.** Orientador: Hilário Cuquetto Mantovani. Co-orientadores: Marisa Vieira de Queiroz e Célia Alencar de Moraes.

Neste trabalho objetivou-se analisar os fatores que interferem na produção de bovicina HC5 por *Streptococcus bovis* HC5 e obter mutantes não produtores de bacteriocina. *S. bovis* HC5 cresceu e produziu bovicina HC5 utilizando várias fontes de carbono e nitrogênio. A maior atividade específica de bovicina HC5 (AU ml^{-1} mg de massa seca $celular^{-1}$) foi obtida em meio contendo glicose na concentração de 16 g l^{-1}, quando *S. bovis* HC5 atingiu a fase estacionária de crescimento. A atividade específica de bovicina HC5 e a produção de biomassa aumentaram aproximadamente 3 vezes quando 0,5 g l^{-1} de extrato de levedura e 1,0 g l^{-1} de Trypticase® foram adicionados ao meio de cultura. *S. bovis* HC5 também foi capaz de produzir bacteriocina em meio contendo soro de leite e em caldo de cana como fonte de carbono. O crescimento de *S. bovis* HC5 foi observado em meio basal, tanto em aerobiose quanto em anaerobiose, mas a produção de bacteriocina foi sempre maior em condições anóxicas. *S. bovis*

apresentou maior taxa de crescimento e rendimento de biomassa quando cultivado em pH 7,0 e à temperatura de 45°C. Entretanto, a produção de bacteriocina foi maior em pH 6,5 e em temperaturas na faixa de 30°C a 39°C. Quando o pH da cultura foi mantido constante durante o crescimento de *S. bovis* HC5, a produção de bacteriocina foi inversamente proporcional à produção de lactato, ao consumo de glicose e ao aumento do pH. Experimentos com cultura contínua com taxa de diluição variando de 0,07 h^{-1} a 1,20 h^{-1}, sem controle de pH, resultaram em fermentação homoláctica e maior produção de bovicina HC5 em taxas de diluição menores. Nesta condição, embora a glicose nunca tenha sido completamente utilizada, observou-se decréscimo no pH da cultura para aproximadamente 5,0. Quando o pH foi mantido em 7,5, fermentação ácido-mista foi observada em taxas de diluição abaixo de 1,20 h^{-1}. A produção de bacteriocina foi maior em taxas de diluição maiores, mas os níveis detectados foram pelo menos 10 vezes menores quando comparado com a condição em que o pH não foi controlado. Nesta condição, glicose residual só foi detectada no fermentador quando a taxa de diluição foi maior que 0,60 h^{-1}. Os resultados obtidos indicam que a produção de bovicina HC5 não está relacionada com o crescimento de *S. bovis* HC5, e pode variar com o pH extracelular e a taxa de crescimento. *S. bovis* HC5 foi transferido por 40 vezes em meio basal e a produção de bovicina HC5 mostrou ser um fenótipo estável. Nenhum plasmídeo foi detectado no genoma de *S. bovis* HC5, indicando que os genes relacionados com a produção de bovicina HC5 podem estar localizados no cromossomo. Trinta mutantes com capacidade reduzida de produzir bovicina HC5 foram obtidos. A presença do vetor utilizado para mutagênese insercional no genoma de alguns mutantes foi confirmada por técnicas moleculares e esses mutantes poderão ser utilizados para a caracterização dos determinantes genéticos envolvidos na síntese de bovicina HC5.

ABSTRACT

CARVALHO, Ana Andréa Teixeira de, D.Sc. Universidade Federal de Viçosa, June, 2009. **Factors that affect bovicin HC5 production and isolation of non-bacteriocin producing mutants.** Adviser: Hilário Cuquetto Mantovani. Co-Advisers: Marisa Vieira de Queiroz and Célia Alencar de Moraes.

This work was performed to investigate the factors that influence bovicin HC5 production by *S. bovis* HC5 and to obtain mutants unable to produce bovicin. *S. bovis* HC5 produced bovicin using a variety of carbon and nitrogen sources. The highest specific activity was obtained in media containing 16 g l^{-1} of glucose, after 16 h of incubation. The peak in cell-free and cell-associated bovicin HC5 activity was detected when *S. bovis* HC5 cultures reached stationary phase. The bovicin HC5 specific activity and bacterial cell mass increased approximately 3-fold when 0.5 g l^{-1} yeast extract and 1.0 g l^{-1}Trypticase® were added to the basal medium. *S. bovis* HC5 cultures were able to produced bovicin HC5 in media containing cheese whey and sugar cane juice as carbon sources. *S. bovis* HC5 grew in basal media, at aerobic and anaerobic conditions, but bacteriocin production was higher under anaerobic conditions. *S. bovis* HC5 grew faster and produced more biomass when cultivated at pH 7.0 and 45ºC, but bovicin HC5

production was higher when the initial pH was 6.5 and the temperatures ranged from 30°C to 39ºC. When the pH was maintained at constant values, bacteriocin production was inversely proportional to lactate production, glucose consumption and the increase in pH. Continuous culture experiments with dilution rates varying from 0.07 to 1.20 h^{-1} under uncontrolled pH resulted in homolactic fermentation and greater bacteriocin production at lower dilution rates. In this latter case, residual glucose was never completely consumed, but the media pH was approximately 5.0. When the pH was maintained at 7.5, mixed-acid fermentation occurred at dilution rates below 1.20 h^{-1}. Bacteriocin production increased at higher dilution rates but was at least 10 times lower when compared with uncontrolled pH conditions. Residual glucose was only detected in the fermentation vessel at dilution rates above 0.60 h^{-1}. These results indicate that bovicin HC5 production is not directly related to *S. bovis* HC5 growth, and can vary depending on extracellular pH and growth rate. *S. bovis* HC5 cells were transferred 40 times in basal media and bovicin HC5 production was a stable phenotype in *S. bovis* HC5. No plasmids were detected in *S. bovis* HC5 genome, indicating that the genes encoding for bovicin HC5 biosynthesis are located in the chromosome. Thirty mutants that showed reduced ability to produce bovicin HC5 were isolated in this study. The presence of the plasmid vector used for insertional mutagenesis in the genome of some mutants was confirmed by molecular techniques and these mutants can be used for characterization of the bovicin HC5 genetic determinants.

INTRODUCTION

Antimicrobial peptides have attracted the attention of several research groups in the last decades due to its industrial application. These antimicrobial substances are produced by several organisms, including insects, plants and animals, as part of their innate immunity, and also play a role in bacterial competition. Antimicrobial peptides are typically characterized as positively charged amphypatic molecules of low molecular mass. Although these molecules vary in their spectrum of activity, inhibitory effects against viruses, bacteria and fungi have been reported.

The antimicrobial peptides produced by bacteria are among the most studied ones due to the simplicity for production and purification and its potential industrial application. The ribossomally synthesized antimicrobial peptides produced by bacteria are called bacteriocins and those produced by lactic acid bacteria (LAB) are the ones more thoroughly characterized. Bacteriocins produced by LAB are mainly active against gram-positive bacteria and can inhibit several food-borne spoilage and pathogenic microorganisms. Reports have also indicated that bacteriocins could be

useful to control bacterial pathogens that cause animal and human infections such as mastitis, gastritis, acne and caries.

Considering the growing interest for commercial application of bacteriocins, the understanding of the factors that influence bacteriocin production is important to enhance peptide yield and to reduce production costs. Moreover, the genetic determinants involved in bacteriocin biosynthesis must be identified and characterized. Previous studies indicate that growth conditions can affect bacteriocin expression and secretion.

Bovicin HC5, a bacteriocin produced by *Streptococcus bovis* HC5 has shown potential for application in food preservation and animal therapy. Bovicin HC5 has been chemically characterized and its spectrum of activity has been determined. Moreover, studies assessing the cytotoxic effects of bovicin HC5 have indicated low toxicity against animal cell lineages. However, the factors affecting bovicin HC5 production have not been fully characterized and its genetic determinants have not yet been identified. In this work, experiments were designed to 1) study the effect of growth conditions on bovicin HC5 production by *S. bovis* HC5 and 2) isolate non-bacteriocin producing mutants using insertional mutagenesis.

CHAPTER 1

LITERATURE REVIEW

1.1 Bacteriocins from lactic acid bacteria

Bacteriocins are extracellularly released, proteinaceous antimicrobial compounds produced by bacteria that vary in structure, biochemical properties, mode of action and spectrum of activity (Cleveland *et al.,* 2001; Cotter *et al.*, 2005). These peptides are ribosomally produced as a pre-peptide that is often modified post-translationally to achieve its biologically active form. The producer strain has immunity against its own bacteriocin, and this immunity can be mediated by a protein that protects the cell against bacteriocin activity or by a transport system that pumps the peptide in the cytoplasm out of the cell (Cleveland *et al.*, 2001; Chatterjee *et al.*, 2005). Several bacteriocins have been characterized and at least some peptides show potential for

application in the food industry and veterinary or human medicine (Delves-Broughton *et al.*, 1996; Grande *et al.*, 2005; Bowe *et al.*, 2006; Carvalho *et al.,* 2007a,b).

Bacteriocins vary in molecular structure, mass, thermostability, mode of action, and genetic determinants. Most peptides produced by lactic acid bacteria (LAB) can be classified into two main classes. The lantibiotics (Class I) are composed of heat-stable antimicrobial peptides of low molecular mass (< 5 kDa) that contain post-translationally modified amino acids. Nisin and subtilin are peptides representatives of this class (Cleveland *et al.*, 2001; Cotter *et al.*, 2005; Deegan *et al.*, 2006). Common post-translational modifications include dehydration of Ser and Thr residues in the precursor peptide to generate unsaturated amino acids, which react intramolecularly with nearby cysteine residues to form cyclic thioethers termed lanthionines and methyllanthionines (McAuliffe *et al.*, 2001; Chatterjee *et al.*, 2005). These thioethers rings are important for bacteriocin activity and stability against proteolysis and thermal inactivation (McAuliffe *et al.*, 2001; Chatterjee *et al.*, 2005; Rink *et al*., 2007).

Lantibiotics also contain other unusual post-translationally modified unsaturated residues named 2,3-didehydroalanine (Dha) and (Z) 2,3-didehydrobutyrine (Dhb) (McAuliffe *et al.*, 2001; Cleveland *et al.*, 2001; Cotter *et al.*, 2005; Chatterjee *et al.*, 2005). It has been suggested that these dehydrated residues might be important for the interaction of the peptide with the cell membrane or spore coat of sensitive microorganisms (Liu and Hansen, 1992; Chan *et al*., 1996). Due to the presence of these modified amino acid residues, the identification of the amino acid sequence of lantibiotics by Edman degradation is often complicated (McAuliffe *et al.*, 2001; Chatterjee *et al.*, 2005). The class II or non-lantibiotic bacteriocins are typically heat stable low molecular mass peptides that do not have post-translationally modified amino

acids and range in size from 37 to 48 residues, such as pediocin and mesentericin (Cotter *et al.*, 2005; Deegan *et al.*, 2006).

The mechanisms by which bacteriocins exert their antimicrobial activity vary among bacterial species. Most peptides form pores on the cell membrane and at least some lantibiotics can also inhibit cell wall biosynthesis or prevent the outgrowth of bacterial endospores (Cleveland *et al.*, 2001; Chatterjee *et al.*, 2005). The inhibitory effect is more pronounced against gram-positive microorganism and can be mediated or not by specific receptors in the cell envelope. Bacteriocin activity can be either bacteriostatic or bactericidal, depending on the structure and biochemical properties of a given peptide (Héchard and Sahl, 2002; Deegan *et al.*, 2006).

Pore formation was the first characterized mode of action. When a bacteriocin form pores on the cytoplasmic membrane of sensitive cells it causes the depolarization of the cell membrane and the loss of intracellular metabolites, such as ATP and cations (Figure 1.1). Some bacteriocins can also inhibit DNA replication and enzyme activity (Breukink *et al.*, 1999; Papagianni, 2003). Even intrinsically resistant microorganisms, such as gram-negative bacteria, can become sensitive to bacteriocins if exposed to treatments that alter the integrity of the outer membrane (Stevens *et al.*, 1991; Ray, 1993).

Although several bacteriocins have been characterized and showed to be effective in controlling pathogenic and food-borne spoilage microorganisms, to date only nisin and pediocin PA-1 have been commercially used (Cotter *et al.* 2005). Nisin was the first bacteriocin approved to be used in foods. This bacteriocin was first described in 1928, but only in 1988 it gained GRAS status (Generally Recognized as Safe). Nisin was approved by the US Food and Drug Agency (FDA) to be used in food products in concentrations ranging from 5.5 to 12.5 mg per kg of food (Siragusa *et al.*,

1999; Cleveland *et al.*, 2001; Cotter *et al.*, 2005; Deegan *et al.*, 2006). Besides nisin, the use of pediocin PA1 in the form of ALTA® 2431 has been covered by several US and European patents (Rodríguez *et al.*, 2002; Drider *et al*., 2006).

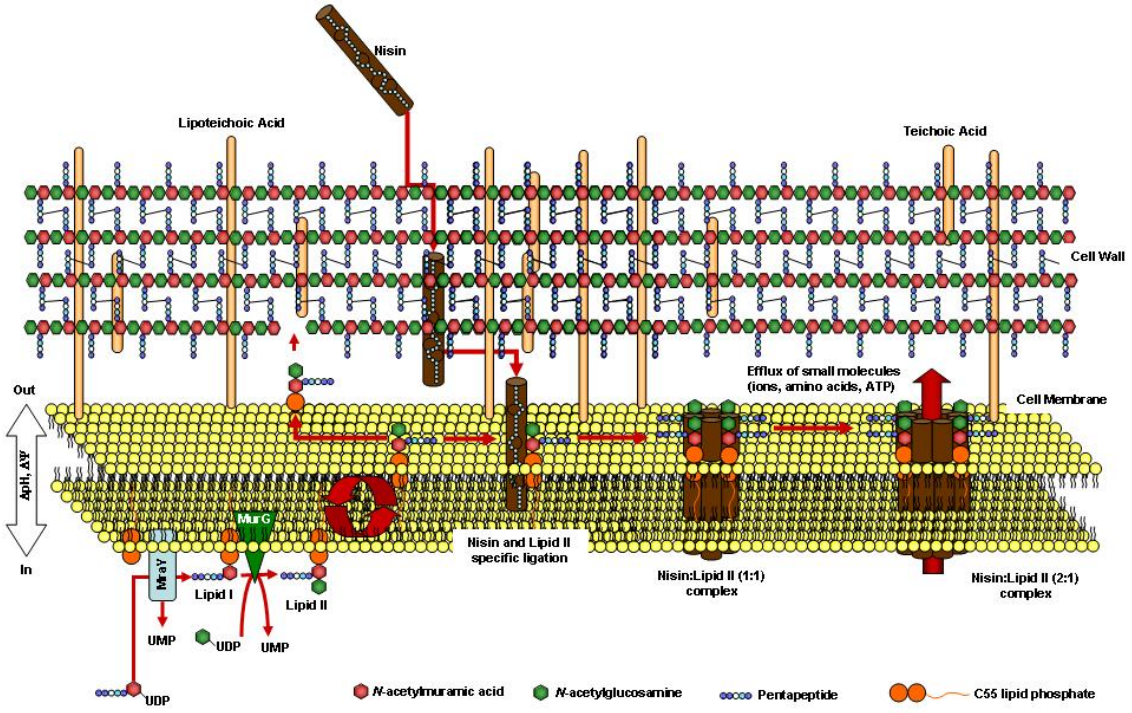

Figure 1.1. A scheme representing the mode of action of nisin. Nisin and some other members of the lantibiotic class of bacteriocins have a dual mode of action involving the binding to lipid II, a membrane-anchored cell wall precursor that is essential for bacterial cell wall biosynthesis. At high concentrations nisin, binds to lipid II in the cell membrane and prevent cell wall biosynthesis. At low concentrations, nisin uses the lipid II as a docking molecule to form pores in the cytoplasmic membrane that cause the loss of intracellular metabolites (e.g. ATP, K^+).

In order for a bacteriocin to be approved for industrial application its effectiveness and safety must be demonstrated. This usually requires biochemical characterization of the peptide and its mode of action, the identification of genetic determinants and the study of potential allergenic and toxicological effects (Cleveland *et al.,* 2001; Rodríguez *et al.,* 2002). In this regard, the characterization of the factors that influence bacteriocin secretion and activity are important to understand the regulation of bacteriocin expression and to maximize its production.

1.2. Factors that affect bacteriocin production by lactic acid bacteria

Bacteriocin production by LAB has been generally associated with the shift from log to stationary phase. Nisin production appears to begin during mid-log phase and reaches its maximum when the cells enter stationary phase (Lejeune *et al.,* 1998). Bacteriocin production also varies according to media composition, and carbon and nitrogen sources are considered major factors affecting cell growth and bacteriocin yield (Kim *et al.*, 2006). Bacteriocin production by LAB is usually enhanced in complex media, such as MRS, due to the fastidious character of these bacteria (Kim *et al.*, 2006). Todorov and Dicks (2006) observed that tryptone and yeast extract were the best nitrogen sources and maltose the best carbon source for bacteriocin production by *Lactobacillus plantarum* strains. Chandrapati and O'Sullivan (1999) demonstrated that nisin expression was induced in *Lactococcus lactis* by lactose or galactose. Kim *et al.* (2006) showed that tryptone were the best nitrogen source for micrococcin GO5 production by *Micrococcus* sp. GO5.

In addition, environmental factors such as pH, temperature and atmosphere of incubation also exerts pronounced influence on growth rate and bacteriocin production

(Nicolas *et al.*, 2004; Neysens and De Vuyst, 2005; Kim *et al.*, 2006). These factors can either influence the growth of the producer strain or affect gene expression and the activity of a particular bacteriocin (Verluyten *et al.*, 2004b). The media pH affects the growth of producer strains and interferes with the stability of bacteriocins by affecting post-translational modification, aggregation, adsorption, proteolysis and the activity of the peptide (De Vuyst *et al.*, 1996; Cheigh *et al.*, 2002). Certain bacteriocins are better produced in conditions of low extracellular pH (Bárcena *et al.* 1998; Guerra and Pastrana, 2003; Hindré *et al.*, 2004). In the case of pediocin AcH, this fact was attributed to the low pH required for post-translational processing of the bacteriocin (Biswas *et al.*, 1991).

The expression of lacticin 481, produced by *Lactococcus lactis*, is induced by the acidic pH resulting from lactic acid accumulation in the growth media or in media artificially acidified (Hindré *et al.*, 2004), and similar results have been reported to nisin and pediocin (Guerra and Pastrana, 2003). As the expression of some bacteriocins appears to be regulated by quorum sensing, it has been proposed that the acidic pH might be related to a cell density signaling. However, more recent studies have shown that at least some bacteriocins might be optimally produced at neutral pH (Settanni *et al.*, 2008).

The influence of incubation temperature on bacteriocin activity and production has been reported to nisin Z (Matsusaki *et al.*, 1996), sakacin A (Diep *et al.*, 2000) and amylovorin 1471 (De Vuyst *et al.*, 1996). Temperature can influence bacteriocin activity, protease activity and cell-bacteriocin or bacteriocin–bacteriocin interaction (Drosinos *et al.*, 2006). High levels of micrococin were obtained when *Micrococcus* sp. GO5 was grown at 37°C (Kim *et al.*, 2006), while nisin Z was optimally produced at 30°C (Matsusaki *et al.*, 1996).

Although most LAB grow better under anaerobic conditions, the production of nisin, amilovorin and pediocin was enhanced when cultures were grown under oxygen-enriched atmospheres (De Vuyst *et al.*, 1996; Cabo *et al.*, 2001; Anastasiadou *et al.*, 2008). Aeration affects bacteriocin production through changes in metabolic pathways that alter growth yield (Neysens and De Vuyst, 2005). Neysens and De Vuyst (2005) showed that amylovorin titers were higher when the producer strain was kept in anaerobic condition under a continuous flow of carbon dioxide.

Typically, the conditions described to improve bacteriocin production may not coincide with the optimum growth condition for the producer strain (Messens *et al.*, 2002; Mataragas *et al.*, 2003; Drosinos *et al.*, 2006). Van den Berghe *et al.* (2006) stated that under optimal growth conditions the consumption of available nutrients and formation of cell mass will be maximal. Less energy will be devoted to maintenance and competition does not become an issue. However, at low growth rates more energy is dedicated to maintenance and the production of antimicrobial substances confers competitive advantage. Therefore, under these conditions bacteriocin production might increase (De Vuyst *et al.*, 1996; Aasen *et al.*, 2000).

Experiments with continuous cultures indicated that growth rate might play an important role in bacteriocin production. Bárcena *et al.* (1998) reported an increase in plantaricin C production by *Lactobacillus plantarum* LL441 at low dilution rates. Higher concentrations of plantaricin C were obtained in carbon-limited continuous cultures kept at pH 5.0 with dilution rates ranging from 0.05 h^{-1} (glucose-limited) to 0.10 or 0.12 h^{-1} (sucrose- and fructose-limited, respectively). When cells grew rapidly in any of these carbon sources, bacteriocin production was abolished (Bárcena *et al.*, 1998). However, in the case of enterocin 1146 (Parente *et al.*, 1997) and divercin (BhugalooVial *et al.,* 1997), higher dilution rates resulted in low bacteriocin levels.

Because the immunity of the producer strain can affects the amount of bacteriocin that is produced, some attempts have been made in order to increase the resistance of the strains to their own peptide. In an earlier work, Kim *et al.* (1998) improved nisin production in *L. lactis* by increasing the expression of NisI, the immunity protein, with a plasmid that carried the immunity genes (Kim *et al.*, 1998). Similar phenotypes were obtained by Quiao *et al.* (1997), whom selected for nisin resistance among nisin-producing strains.

1.3. Genetics of bacteriocin production

Bacteriocins are gene encoded peptides that can be found in the chromosome (subtilin) or in mobile genetic elements, such as plasmids (lacticin 3147) and conjugative transposons (nisin) (Chatterjee *et al.*, 2005). Several proteins are involved in bacteriocin biosynthesis, including regulatory proteins, proteases, transport proteins and immunity proteins (Figure 1.2). The genes encoding these proteins are commonly located close to the structural gene and are organized in one or more operons, which may or not be divergently transcribed (Chatterjee *et al.*, 2005).

Nisin, the most studied bacteriocin, has 11 genes involved in its biosynthesis: *nis*A (structural peptide); *nis*B (modification); *nis*T (translocation); *nis*C (modification); *nis*I (immunity); *nis*P (protease); *nis*RK (regulation); and *nis*FEG (immunity). These genes are organized in three operons: the *nis*A and *nis*F operon, which are induced by nisin, and the *nis*R operon which is constitutive (Figure 1.2). The synthesis of nisin is regulated by a quorum sensing mechanism, where nisin acts as the induction factor (Kuipers *et al.*, 1998; Chatterjee *et al.*, 2005). The NisRK proteins are part of a two-component regulatory system, NisR being the response regulator and NisK a histidine

kinase (Figure 1.2). When sub inhibitory amounts of nisin are present in the growth media, the NisK protein is activated and the genes related to nisin production are activated (Figure 1.2). Nisin transcription can be induced by the addition of nisin to the culture medium and the level of induction obtained seems directly related to the level of nisin added (Kuipers *et al.,* 1993; 1995).

Bacteriocins are typically synthesized as a pre-peptide containing a N-terminal leader sequence. This leader sequence may function to maintain the peptide inactive inside the producer strain and could also serve for recognition by the transport and modification machinery (Van Der Meer *et al.*, 1994). The leader sequence is removed by NisP while the bacteriocin is transported to the extracellular medium to achieve its active form (Figure 1.2) (McAuliffe *et al.*, 2001). Structural similarities among leader peptides from bacteriocins belonging to the same class have been observed, suggesting that this part of the molecule plays an integral role in lantibiotic biosynthesis (Kaletta and Entian, 1989).

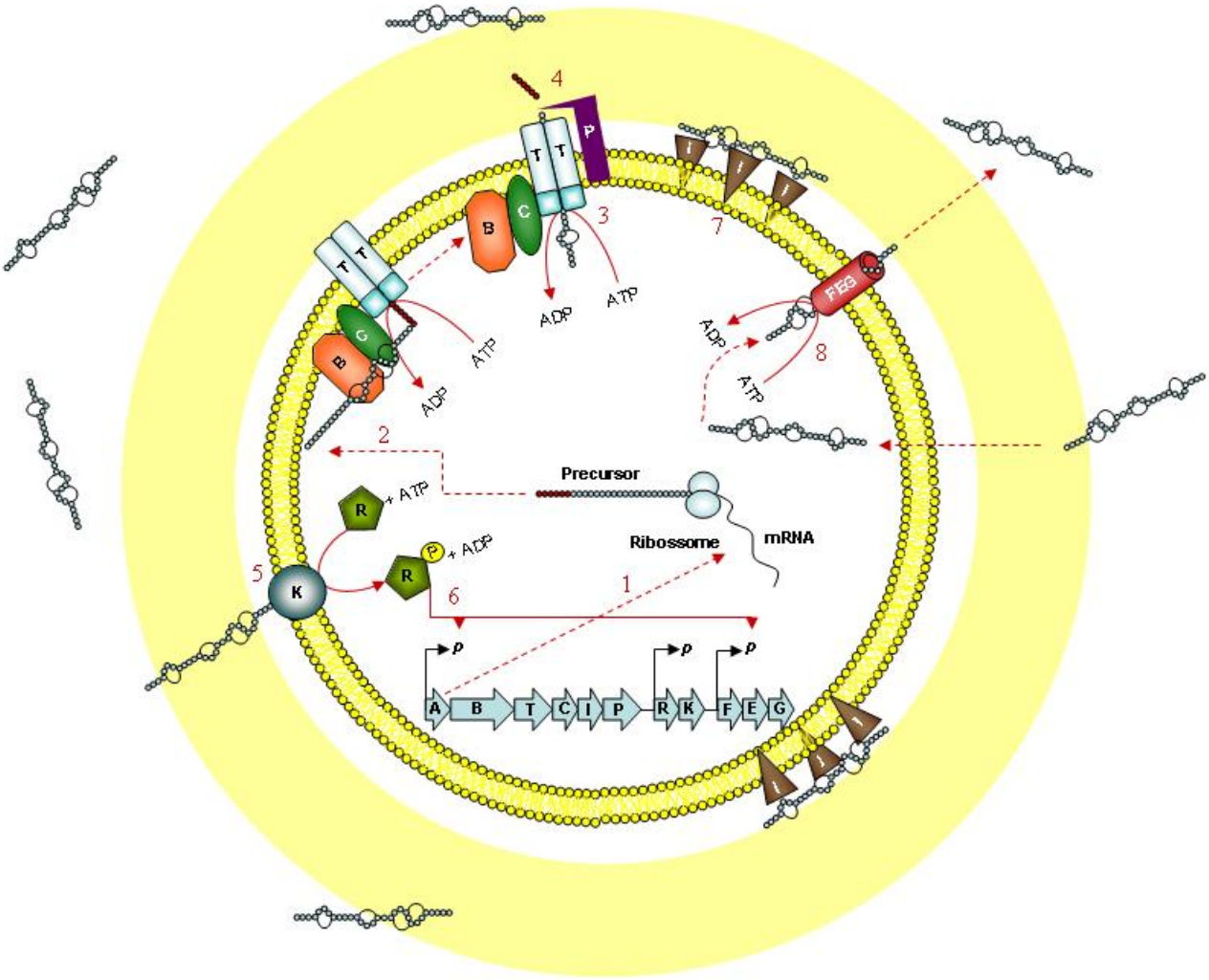

Figure 1.2. A scheme showing the major events during nisin biosynthesis. Nisin is transcribed from *nis*A (1) and NisB and NisC modify the prepeptide (2). The post-tanslationally modified peptide is translocated across the membrane by the exporter NisT (3). The precursor is extracellularly processed by NisP, releasing the mature nisin (4). Regulatory protein NisK senses the presence of nisin and undergoes autophosphorylation (5). The phosphate-group is transferred to NisR, the response regulator, which activates transcription of *nis*ABTCIP and *nis*FEG (6). The *nis*RK operon is constitutive. The NisI (7) and NisFEG (8), are part of the immunity system that prevents nisin from being bactericidal against the producer cell. P: promoter region. For details see the text.

Several lantibiotics, including subtilin, epidermim and lacticin 481 have been genetically characterized. Although characteristics among bacteriocins vary regarding gene distribution and transcriptional organization, peptides from the same class usually share similar characteristics (Chatterjee *et al.*, 2005). Comparison of gene clusters indicated the presence of conserved ORFS encoding similar functions, as demonstrated for imunity proteins (McAuliffe *et al.*, 2001). In fact, the organization of some operons encoding for lantibiotics might be similar to those involved in the biosynthesis of class II bacteriocins. Similar to the lantibiotics, class II bacteriocins have the genes involved in bacteriocin synthesis organized in one to three operon-like structures. However, class II bacteriocins operons do not harbor genes involved in peptide modification (Ennahar *et al.*, 2000).

The techniques that have been used to characterize bacteriocins at molecular level includes site-directed mutagenesis (Oppegård *et al.*, 2007), PCR (Cookson *et al.*, 2004), the use of degenerate primers (Wirawan *et al.*, 2006), expression systems (Rodriguez *et al.*, 2003) and gene inactivation (Fontaine *et al.*, 2007). Liu and Hansen (1992) and Oppegård *et al.* (2007) used site-directed mutagenesis to study structural aspects of subitlin and lactococcin G and Enterocin 1071, respectively, and their effect on antimicrobial activity.

To study the function of the genes involved in bacteriocin biosynthesis, several expression systems for production of lantibiotics have been constructed. Gutiérrez *et al.* (2005) cloned and studied the expression in *Escherichia coli* of the gene enconding for enterocin P, a bacteriocin produced by *Enterococcus faecium* P13. The enterocin A gene, produced by *E. faecium* PLBC21, was cloned in *Lactococcus lactis* to address the expression and production of these bacteriocin in the same host. *L. lactis* was able to

produce both enterocin A and nisin and the production of enterocin was greater in *L. lactis* than in the native host (Martín *et al.*, 2007).

In a previous work, Maguin *et al.* (1996) developed a vector named pGh9:ISS1 that has been used to inactivate several genes of different strains of LAB. This vector is a thermosensitive plasmid that contains the insertion sequence ISS1. A high-frequency of transposition (at least 0.5 % in *L. lactis*) has been obtained for this system, which has allowed efficient gene inactivation and direct cloning of DNA surrounding the insertion. Ward *et al.* (2001) applied the vector to identify the biosynthetic genes encoding for hyaluronic acid capsule in *Streptococcus uberis*. And Mora *et al.* (2004) used the pGh9:ISS1 system to characterize the urease gene cluster of *Streptococcus thermophilus*. In a more recent work, Fontaine *et al.* (2007) utilized the pGh9:ISS1 vector to characterize the genes involved in quorum-sensing regulation of bacteriocin production by *Streptococcus thermophilus*.

1.4. Bovicin HC5

Bovicin HC5 is a bacteriocin produced by *Streptococcus bovis* HC5, a rapidly growing gram-positive ruminal bacterium that has few nutritional requirements (Mantovani *et al.*, 2002). Bovicin HC5 has a molecular mass of 2440 Da, shows stability at high temperatures and acidic pH and is inactivated by trypsin (Mantovani *et al.* 2002; Houlihan *et al.* 2004). The amino acid sequence of this bacteriocin was partially characterized (VGXRYASXPGXSWKYVXF) and showed structural similarities with the lantibiotics. The unidentified amino acid residues (designated by X) were positioned at locations similar to the dehydroalanines found in nisin, a Class I (lantibiotic) bacteriocin (Mantovani *et al*., 2002).

The antimicrobial activity of bovicin HC5 also resembles the lantibiotics, causing the loss of intracellular potassium from sensitive cells (Mantovani *et al.*, 2002; Houlihan *et al.*, 2004; Mantovani and Russell, 2008). Bovicin HC5 has a broad spectrum of activity, being inhibitory against several strains of food-borne and spoilage microorganisms, including *Listeria monocytogenes*, *Clostridium tyrobutyricum*, *Bacillus cereus, Bacillus thuringiensis*, *Alicyclobacillus acidoterrestris* and strains of LAB, such as *Streptococcus, Lactococcus, Enterococcus* and *Lactobacilus* (Mantovani *et al.*, 2002; Mantovani and Russell, 2003; Carvalho *et al.*, 2007ab; Carvalho *et al.*, 2008).

Because of its biochemical characteristics and activity at low pH, bovicin HC5 might be suitable to control thermoacidophilic spoilage bacteria in heat-treated acidic drinks. Carvalho *et al.* (2007ab) showed that bovicin HC5 was bactericidal against *B. cereus*, *B. thuringiensis* and *C. tyrobutyricum* in mango pulp, and the inhibitory effect was even more pronounced at acidic conditions (Carvalho *et al.*, 2007ab). Bovicin HC5 also reduces the outgrowth of endospores from *B. cereus* and *B. thuringiensis* in acidic mango pulp (Carvalho *et al.*, 2007a). These latter results indicated that bovicin HC5 was as effective as nisin to control *Bacillus* and *Clostridium* strains in mango pulp (Carvalho *et al.*, 2007ab).

When bovicin HC5 was tested in mango pulp against *A. acidoterrestris*, a thermoacidophilic microorganism, the bacteriocin showed bactericidal effect against vegetative cells and sporicidal activity (Carvalho *et al.*, 2008). The *D*-values of *A. acidoterrestris* spores that had been heat treated in acidic mango pulp decreased more than 90% in the presence of bovicin HC5, compared to untreated controls (Carvalho *et al.*, 2008). Recently, Souza (2008) demonstrated that bovicin HC5 was also effective against spores and vegetative cells of *A. acidoterrestris* in different fruit juices. These

results suggest that bovicin HC5 could be useful to prevent spoilage of acidic fruit products by spore-forming bacteria.

Bovicin HC5 has also potential for use in veterinary medicine. This bacteriocin was effective against strains of *Stapphylococcus aureus*, coagulase-negative *Stapphylococcus* sp., *Streptococcus agalactiae, Streptococcus bovis* and *Streptococcus uberis* isolated from cows with mastitis (Pinto, 2007). The activity against these animal pathogens appears to be bactericidal. The combined use of bovicin HC5 with antibiotics or others bacteriocins that are effective against mastitis-causing pathogens may represent an alternative strategy to control bovine mastitis.

Bovicin HC5 has been recently used to manipulate ruminal fermentation in vitro and might be useful to improve feed efficiency in cattle (Lima *et al.* 2009). Bovicin was as effective as the feed additive monensin to inhibit the deamination of mixed ruminal bacteria *in vitro*. Considering that the European Union banned the use of monensin as a growth-promoting antibiotic (Russell and Houlihan, 2003), bovicin HC5 could be considered a potential feed additive for cattle.

Due to its potential commercial applications, the effectiveness, production and safety of bovicin HC5 must be fully understood. This requires the investigation of genetic determinants, mode of action, efficacy, spectrum of activity and allergenic and toxicological effects (Cleveland *et al.* 2001; Rodríguez *et al.* 2002). Even though bovicin HC5 has been characterized as an effective and broad-spectrum bacteriocin, the genetic determinants involved in bovicin HC5 biosynthesis are yet to be elucidated. Further studies are also needed to determine the cytotoxic and allergenic potential of bovicin HC5 against mammalian cells. Although bovicin HC5 is produced by *Streptococcus bovis,* and some *S. bovis* strains can cause infection in humans, studies

indicate that bovine *S. bovis* strains are genetically and physiologically distinct from human isolates (Whitehead and Cotta, 2000; Jarvis *et al.,* 2000; Kurtovic *et al.,* 2003).

1.5. References

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CHAPTER 2

The effect of carbon and nitrogen sources on bovicin HC5 production by *Streptococcus bovis* HC5[*]

2.1. Abstract

The aim of this study was to investigate the effect of media composition and agroindustrial residues on bovicin HC5 production by *Streptococcus bovis* HC5. Batch cultures of *S. bovis* HC5 were grown in basal medium containing different carbon and nitrogen sources. The activity of cell-free and cell-associated bovicin HC5 was determined in culture supernatants and acidic extracts obtained from cell pellets, respectively. *S. bovis* HC5 produced bovicin using a variety of carbon and nitrogen sources. The highest specific activity was obtained in media containing 16 g l^{-1} of glucose, after 16 h of incubation. The peak in cell-free and cell-associated bovicin HC5

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activity was detected when *S. bovis* HC5 cultures reached stationary phase. The bovicin HC5 specific activity and bacterial cell mass increased approximately 3-fold when yeast extract and Trypticase® (0.5 and 1.0 g l^{-1}, respectively) were added together to the basal medium. *S. bovis* HC5 cultures produced bovicin HC5 in cheese whey and sugar cane juice and maximal volumetric productivity was obtained after 12 h of incubation. These results demonstrated that *S. bovis* HC5 is a versatile lactic acid bacterium that can utilize several carbon and nitrogen sources for bovicin HC5 production. This bacterium could be a useful model to study bacteriocin production in the rumen ecosystem. The use of agroindustrial residues as carbon sources could have an economical impact on bovicin HC5 production. To our knowledge, this is the first report to show the use of sugar cane juice for bacteriocin production by lactic acid bacteria.

Keywords: bacteriocin production, bovicin HC5, sugar cane juice, cheese whey, media composition

2.2. Introduction

Lactic acid bacteria (LAB) can produce several antimicrobial compounds (e.g. hydrogen peroxide, organic acids and bacteriocins) that inhibit spoilage and pathogenic microorganisms (Deegan *et al.,* 2006). Bacteriocins produced by LAB have received great attention because of their use as 'natural' food preservatives and an increased demand for less-processed and microbiologically safe food products (Gálvez *et al.,* 2007). Nisin is one of the most studied bacteriocins due to its spectrum of activity and potential for industrial and medical applications (Delves-Broughton *et al.,* 1996; Cleveland *et al.,* 2001). However, several reports have demonstrated that some nisin

sensitive bacteria can rapidly become nisin-resistant (Crandall and Montville, 1998; Mantovani and Russell, 2001; Naghmouchi *et al.,* 2007). The development of nisin-resistant strains has increased the interest in studying other bacteriocins with similar spectrum of activity that show stability to heat and acidic conditions.

Bovicin HC5 is a bacteriocin produced by *Streptococcus bovis* HC5 that inhibits the growth of several foodborne and spoilage microorganisms, including *Listeria monocytogenes* (Mantovani and Russell, 2003a), *Bacillus cereus* and *B. thuringiensis* (Carvalho *et al.,* 2007a) and certain species of *Clostridium* (Flythe and Russell, 2004; Carvalho *et al.,* 2007b). Bovicin HC5 is a small pore-forming peptide with a unique amino acid sequence that shows similarity to the lantibiotics (Mantovani *et al.,* 2002; Mantovani and Russell, 2008). It has stability to high temperatures and acidic pH (Mantovani *et al.* 2002; Houlihan *et al.,* 2004), and resistance development to bovicin HC5 has not yet been described. Because of its characteristics, bovicin HC5 could also have potential for industrial application (Russell and Mantovani, 2002; Diez-Gonzalez, 2007).

Considering that *S. bovis* HC5 is an aerotolerant anaerobe with very rapid growth rates and simple nutritional requirements (Wolin *et al.,* 1959; Russell and Robinson, 1984), the fermentation processes for bovicin HC5 production could be performed by using low cost substrates and in a short period of time. Previous work indicated that growth conditions could affect bovicin HC5 production and continuous culture experiments indicated that antimicrobial activity was inversely related to the glucose consumption rate (Mantovani and Russell, 2003b). These earlier results suggested that the optimization of the growth conditions could improve bovicin HC5 production. The following experiments aimed to: (i) determine the effect of carbon and nitrogen sources on bovicin HC5 production by *Streptococcus bovis* HC5 and (ii)

examine the potential for using sugar cane juice and cheese whey as alternative substrates for bacteriocin production.

2.3. Materials and methods

2.3.1. Microorganisms and growth conditions

Streptococcus bovis HC5 was originally isolated from a grain fed cow (Mantovani *et al.*, 2001) and cultivated as previously described (Mantovani and Russell, 2003). *S. bovis* HC5 was grown in basal media containing (per liter): 292 mg K_2HPO_4, 292 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4.7H_2O$, 64 mg $CaCl_2.2H_2O$, 500 mg cysteine hydrochloride, 1 g Trypticase®, 0.5 g yeast extract and 4 g Na_2CO_3. The medium was prepared anaerobically under an O_2-free carbon dioxide flux and the final pH was adjusted to 6.5 with NaOH (1 mol l^{-1}). Growth and maximal optical densities were determined at 600 nm (OD_{600nm}) in a Spectronic $20D^+$ (Thermoelectron, Madison, WI). The indicator organism, *Alicyclobacillus acidoterrestris* DSMZ 2498, was grown at 40ºC in *Alicyclobacillus acidoterrestris* medium (AAM), described by Yamazaki *et al.* (2000).

2.3.2. Activity of bovicin HC5

Free bovicin HC5 and cell-associated bacteriocin were determined in the cell-free supernatant and in the acidic extract obtained from cell pellets, respectively. *S. bovis* HC5 cells were harvested by centrifugation (9000 *x g*, 4ºC, 15 min) and the culture supernatant was used to determine the activity of free bovicin HC5. The cell pellet was washed (9000 *x g*, 4ºC, 15 min) in 10 ml of sodium phosphate buffer (5

mmol l^{-1}, pH 6.7) and re-suspended in a volume of acidic NaCl solution (100 mmol l^{-1}, adjusted to pH 2.0 with 1 mol l^{-1} HCl) that corresponded to 5% of the initial culture volume. Cell suspensions were incubated under agitation (approximately 150 rev min^{-1}) for 2 h at room temperature, followed by centrifugation (9000 *x g*, 4ºC, 15 min) and determination of bacteriocin activity in the cell-free extract. Preparations containing bovicin HC5 were serially diluted (2-fold increments) with NaCl solution (100 mmol l^{-1}, pH 2.0) and tested for antimicrobial activity against *A. acidoterrestris* DSMZ 2498 by using the agar well diffusion technique described by Hoover and Harlander (1993). One arbitrary unit (AU, expressed per ml or cell dry mass) was defined as the reciprocal of the highest dilution that showed a zone of inhibition with at least 5 mm in diameter. When the determination of optical density was not possible due to the turbidity of the media containing sugar cane juice or cheese whey, bovicin activity was expressed as volumetric productivity (AU ml^{-1} h^{-1}).

2.3.3. Effect of carbon and nitrogen sources on bovicin HC5 production

Carbon sources preferentially used by *S. bovis* strains were previously described by Russell and Robinson (1984) and used in this study. The carbon sources tested were glucose, lactose, maltose, mannose, sucrose and cellobiose at concentrations of 4 and 8 g l^{-1}. Batch cultures (3% inoculum, v/v) were grown in anaerobic basal media (50 ml) and kept incubated for approximately 16 h at 39ºC and activity of bovicin HC5 was tested in the supernatant and in the cell extract.

The carbon source that allowed highest bovicin HC5 activity was further tested to evaluate the effect of concentration on bacteriocin production. The culture was grown for 16 h and the bovicin HC5 activity in the culture supernatant and in the cell-free

extract was determined for each concentration as described above. In each case, the pH and the OD_{600nm} were also determined.

Sugar cane juice and cheese whey were also tested as carbon sources for bacteriocin production by *S. bovis* HC5. Batch cultures were carried out in sealed anaerobic serum bottles (50 ml) or in 500 ml fleaker beaker flasks (Corning) that were continuously purged with O_2-free carbon dioxide. Cheese whey was reconstituted in water, heat-sterilized (121°C, 15 min) and added to 2-fold concentrated basal media (1:1 ratio, v/v). The final lactose concentration was approximately 24 g l^{-1}. Sugar cane juice (16 ° Brix) was extracted from ground sugar cane, heat-sterilized (121°C, 15 min) and mixed with basal media (1:1 ratio, v/v). The final sugar concentration was approximately 100 g l^{-1}. Samples (20 ml) were taken at time intervals (0, 12, 24 and 48 hours) and tested for bacteriocin activity as previously described. Volumetric bacteriocin productivity (Qp, expressed as AU ml^{-1} h^{-1}) was determined from the difference in bovicin activity in relation to the time (h).

To study the effect of different nitrogen sources on bovicin production, the basal medium, added of glucose at 16 g l^{-1}, was supplemented with each different nitrogen source (1.5 g l^{-1}). The nitrogen sources tested were yeast extract, Trypticase®, meat extract, soy peptone, meat peptone, casein peptone, ammonium sulfate. After 24 h of incubation at 39ºC, the final optical densities, culture pH value and bacteriocin activity in the culture supernatant and in the cell extract were determined.

2.3.4. Other analyses

Bacterial dry weight was determined by growing *S. bovis* in basal media containing glucose at 16 g l^{-1} (18 h at 39ºC) and washing the cells twice in phosphate

buffer (5 mM, pH 6.5); the pellet was concentrated four times and aliquots of 5 ml were dried at 105ºC up to constant weight. The relationship between optical density (600 nm) and cell dry mass was 360 mg cell dry mass liter^{-1} turbidity unit^{-1}.

Glucose and fermentation acids in cell-free supernatant were analyzed by high performance liquid chromatography (HPLC, Bio-Rad HPX-87H organic acid column). The sample size was 20 µl, the eluant was 0.005 mol l^{-1} H_2SO_4, the flow rate was 0.7 ml min^{-1} and the column temperature was 60ºC.

2.3.5 Statistical methods

All experimental determinations were performed in three replicates and the mean, standard deviation and coefficients of variation were computed. The coefficients of variation were always less than 10 %. When error bars are given in the figures, they refer to the standard deviation.

2.4. Results

2.4.1. Influence of carbon sources on bovicin HC5 production

Batch cultures of *S. bovis* HC5 grew rapidly in basal medium containing mono- or disaccharides, and cell mass production increased as the sugar concentration was doubled (Table 2.1). However, the release of bovicin HC5 in the cell-free supernatant could only be detected in cultures grown in media containing glucose, sucrose or lactose. Cell-associated bovicin HC5 could be extracted from *S. bovis* HC5 cells grown in all the carbon sources tested in this study, but glucose was the preferred sugar for

bovicin production (Table 2.1). Among the disaccharides tested, the highest activity of cell-associated bovicin HC5 was detected in cellobiose-grown cultures (Table 2.1). Based on these results, glucose was chosen for further characterization of its effect on bovicin HC5 production.

Table 2.1. Influence of carbon sources on cell growth and bovicin HC5 production by *S. bovis* HC5

Carbon source	Concentration ($g\ l^{-1}$)	Final pH	Cell dry mass ($mg\ ml^{-1}$)	Bovicin HC5 specific activity ($AU\ ml^{-1} mg^{-}$ dry cell $mass^{-1}$)	
				Cell-free	Cell-associated
Glucose	4	6.18	0.84	190	3048
	8	5.39	0.94	1702	5446
Sucrose	4	6.09	0.72	-	444
	8	4.52	1.08	592	1185
Mannose	4	5.92	0.45	-	1422
	8	4.75	1.30	-	1970
Maltose	4	6.10	0.39	-	410
	8	5.48	1.59	-	805
Cellobiose	4	5.98	0.59	-	1084
	8	5.67	0.54	-	4740
Lactose	4	5.93	0.52	-	1230
	8	5.59	1.04	615	1230

- Inhibitory activity was not detected

When glucose (up to 160 $g\ l^{-1}$) was added to the growth medium, an increase in cell mass and a decrease in culture pH were observed until the sugar concentration was 16 $g\ l^{-1}$ (results not shown). This increase in cell mass coincided with a greater production of cell-free and cell-associated bovicin HC5 (Figure 2.1). When glucose concentration was 16 $g\ l^{-1}$, *S. bovis* HC5 grew with a specific growth rate of 0.85 h^{-1} and glucose was never completely consumed (Figure 2.2a). Bovicin HC5 production reached its peak after *S. bovis* HC5 cultures had reached stationary phase (Figure 2.2b). Cell-free bovicin HC5 specific activity was approximately 2500 AU ml^{-1} mg cell dry $mass^{-1}$ while the cell-associated activity was around 4 times greater after 16 and 24 h of incubation. Cultures grown at higher (40 and 160 $g\ l^{-1}$) sugar concentrations showed

decreased optical densities (results not shown) and the production of bovicin HC5 was drastically reduced (Figure 2.1).

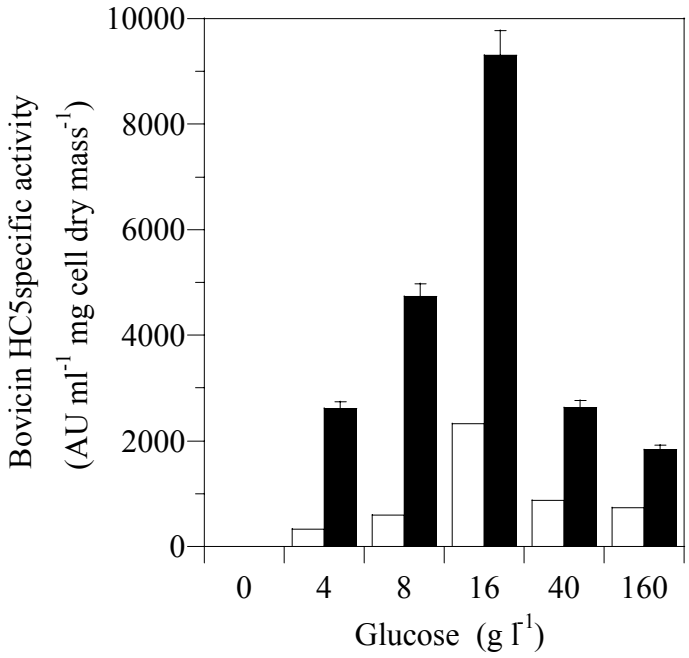

Figure 2.1. The effect of glucose on bovicin HC5 specific activity. *S. bovis* HC5 was inoculated into basal medium added with increasing amounts of glucose (0 to 160 g l^{-1}) and incubated at 39ºC for 16 h. Bovicin HC5 activity was determined in cell-free culture supernatants (open bars) and in extracts from *S. bovis* cells treated with acidic NaCl (closed bars).

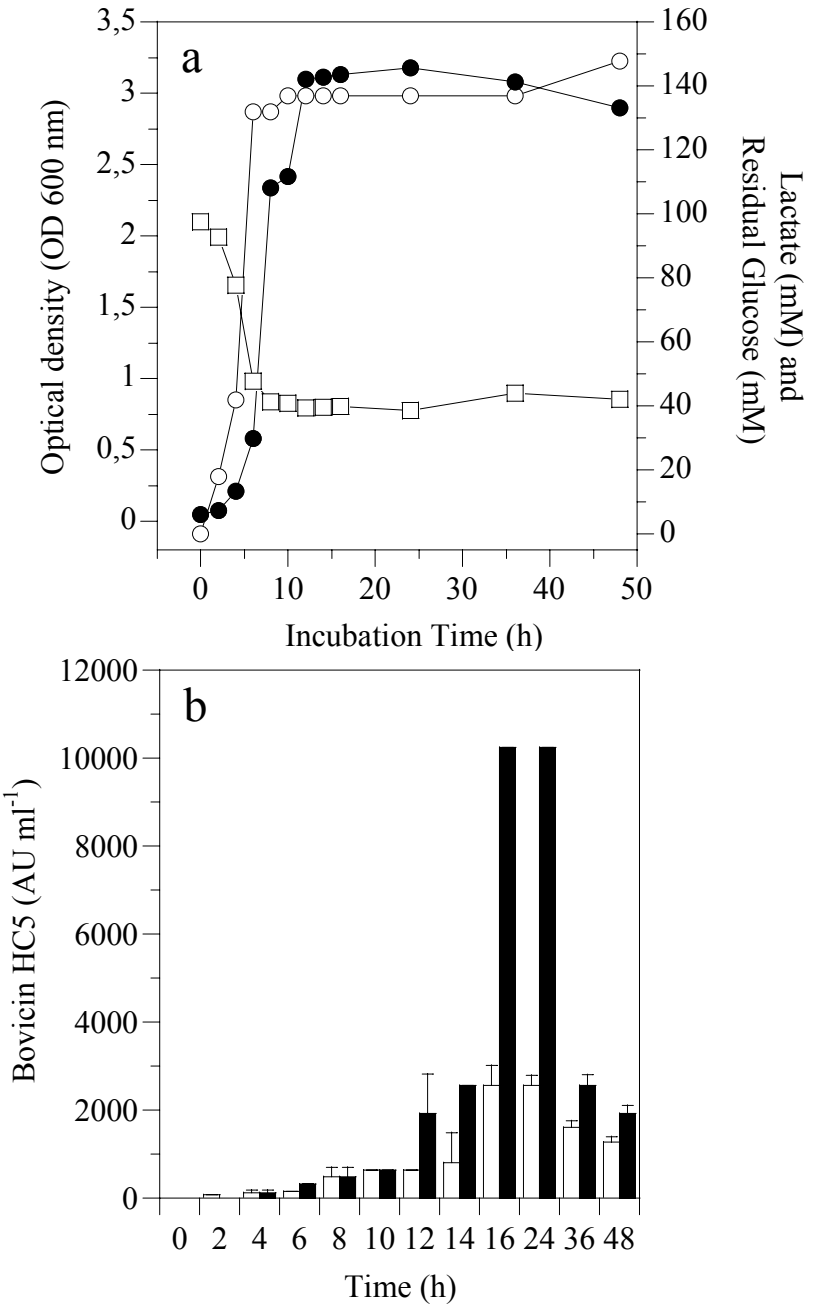

Figure 2.2. Growth and bovicin production by *S. bovis* HC5 cultivated in basal media containing glucose (16 g l^{-1}) as sole carbon source. (a) Growth of *S. bovis* in basal media(open circles), lactate accumulation (closed circles) and residual glucose (open squares). (b) Activity of cell-free (open bars) and cell-associated (closed bars) bovicin HC5 determined at different time intervals.

2.4.2. Influence of nitrogen sources on bovicin HC5 production

When *S. bovis* HC5 was cultivated in basal medium that lacked yeast extract and Trypticase® and was added of different nitrogen sources (at 1.5 g l^{-1}), a range of cell mass and bovicin HC5 specific activities were obtained (Table 2.2). The amount of bovicin HC5 detected in the cell extracts was always higher than in the culture supernatants, except when casein peptone was used as the nitrogen source (Table 2.2). The specific activity of cell-free bovicin varied 10-fold among nitrogen sources, while the cell-associated activity varied approximately 24 fold. When ammonium sulfate was used as the single nitrogen source, *S. bovis* HC5 did not grow well and produced little bacteriocin (Table 2.2).

Table 2.2. Influence of nitrogen sources on cell growth and bovicin HC5 production by *S. bovis* HC5

Nitrogen source	Concentration (g l^{-1})	Final pH	Cell dry mass (mg ml^{-1})	Bovicin HC5 specific activity (AU $ml^{-1}mg^{-}$ dry cell $mass^{-1}$)	
				Cell-free	Cell-associated
Yeast extract	1.5	4.04	0.88	827	2909
Trypticase®	1.5	4.55	0.95	674	1347
Soy peptone	1.5	4.37	0.87	368	552
Meat peptone	1.5	4.66	1.04	769	1230
Casein peptone	1.5	4.33	1.03	466	388
Ammonium sulfate	1.5	5.11	0.36	222	-
Trypticase plus yeast extract	1.0 + 0.5	4.09	1.10	2327	9310

- Inhibitory activity was not detected

When media were added of extra Trypticase® and yeast extract (up to 10 g l^{-1}) a positive effect on bacterial cell mass production was observed (data not shown). However, production of bovicin HC5 and cell mass accumulation was not always associated. The production of bovicin by *S. bovis* HC5 was stimulated by yeast extract

or Trypticase®, but the specific activity of bovicin HC5 decreased if the concentration of yeast extract and Trypticase® was greater than 5 g l^{-1} and 7 g l^{-1}, respectively (Figure 2.3 and Figure 2.4). Interestingly, when optimal concentrations (7 g l^{-1} Trypticase and 1 g l^{-1} yeast extract) of both substrates were added to basal media, an increase in cell mass was observed (1.40 mg ml^{-1} cell dry mass), but the amount of bacteriocin produced was less than observed in basal media alone (data not shown).

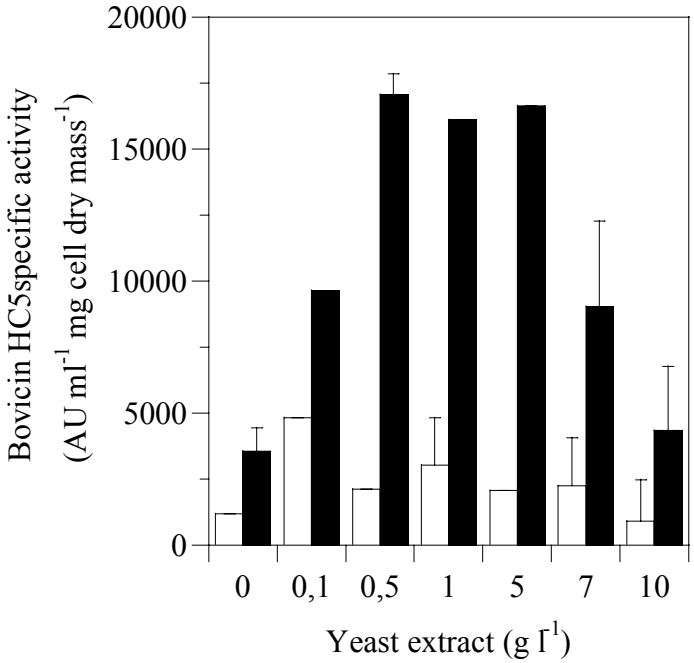

Figure 2.3. The effect of yeast extract on bovicin HC5 specific activity. Basal medium containing 1 g l^{-1} Trypticase® was added of increasing yeast extract concentrations (0 to 10 g l^{-1}) and bovicin HC5 activity in the culture supernatant (open bars) and in the cell extract (closed bars) were determined by the agar well diffusion assay using *A. acidoterrestris* DSMZ 2498 as the indicator organism. *S. bovis* HC5 cultures were incubated anaerobically for 24 h at 39ºC.

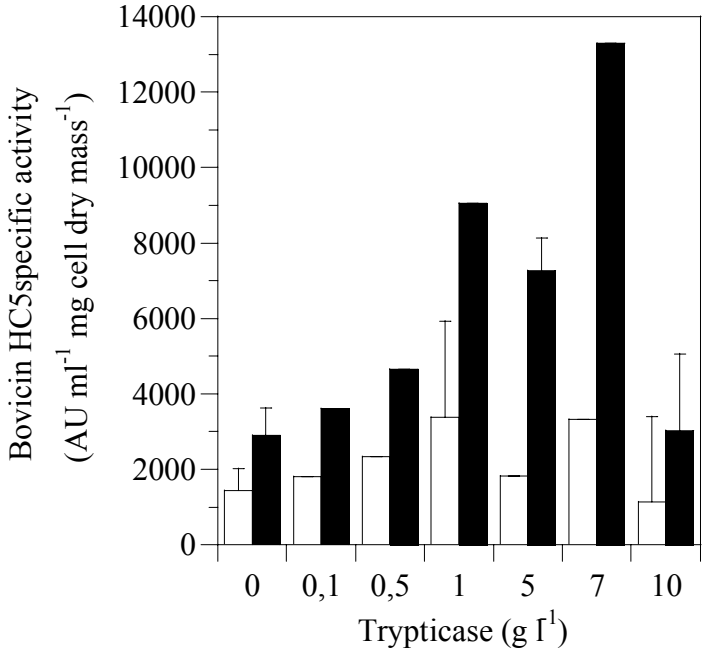

Figure 2.4. The effect of Trypticase® on bovicin HC5 specific activity. Basal medium containing 0.5 g l^{-1} of yeast extract and added of increasing Trypticase® concentrations (0 to 10 g l^{-1}) and bovicin HC5 activity in the culture supernatant (open bars) and in the cell extract (closed bars) were determined by the agar well diffusion assay using *A. acidoterrestris* DSMZ 2498 as indicator microorganism. *S. bovis* HC5 cultures were incubated anaerobically for 24 h at 39ºC.

2.4.3. Use of sugar cane juice and cheese whey for bovicin production

Since *S. bovis* HC5 could use sucrose and lactose for bovicin HC5 production, we tested sugar cane juice and cheese whey as potential abundant, low-cost substrates for bacteriocin production. Batch cultures grown in sealed anaerobic bottles had more cell-free bacteriocin than cultures that were continuously flushed with CO_2 for both substrates (Table 2.3). However, the amount of cell-associated bovicin HC5 was always greater than cell-free, regardless of the condition of incubation, at 12 or 24 h of incubation. In most cases, maximal volumetric productivity was obtained after 12 h of incubation, and sugar cane juice was the substrate that yielded the highest bovicin HC5 activity (Table 2.3).

Table 2.3. Volumetric productivity (Qp) of bovicin HC5 in media containing sugar cane juice and cheese whey

Incubation time (h)	Substrate							
	Sugar Cane Juice (AU ml^{-1} h^{-1})				Cheese Whey (AU ml^{-1} h^{-1})			
	Under CO_2 flux		Sealed bottles		Under CO_2 flux		Sealed bottles	
	CF	CA	CF	CA	CF	CA	CF	CA
12	53	2560	736	2560	53	2133	426	853
24	53	1706	67	1706	160	853	106	640
48	26	10	53	426	53	33	53	320

CF – Cell-free bovicin HC5
CA – Cell-associated bovicin HC5

2.5. Discussion

Lactic acid bacteria (LAB) have been exploited as a major source of a variety of antimicrobial peptides with potential commercial applications (Cleveland *et al.*, 2001;

Gálvez *et al.,* 2007). LAB are fastidious organisms that often require several organic factors (e.g. amino acids, vitamins) for growth. In order to circumvent their nutritional limitations and stimulate the production of bacteriocins and other metabolites with applied interest, LAB are frequently cultivated in complex media (Møretrø *et al.,* 2000; Kim *et al.*, 2006). However, the recovery and purification of bacteriocins can be complicated and costly if the peptide of interest is secreted in media containing contaminating proteins and peptides (Carolissen-Mackay *et al.,* 1997; Papagianni *et al.,* 2007).

Nonetheless, many *S. bovis* strains can use ammonium salts as their sole nitrogen source (Wolin *et al.,* 1959) and simple media can often support *S. bovis* growth and the production of useful metabolites. Bergey´s Manual of Determinative Bacteriology defines *Streptococcus bovis* as "the least nutritionally fastidious species" among streptococci, without a "requirement for any specific amino acid" (Holt *et al.,* 1984) and *S. bovis* HC5, a rapidly growing, gram-positive ruminal bacterium, produces a bacteriocin (bovicin HC5) with broad spectrum of activity (Mantovani *et al.*, 2002). Although *S. bovis* HC5 secretes bovicin into the supernatant when the culture pH is low, much of its antimicrobial activity remains cell-associated (Houlihan and Russell, 2006; Xavier *et al.*, 2008). Because bovicin HC5 can be released from the producer cells with acidic NaCl, purification can be attained in a single chromatographic step using C-18 reversed-phase columns (Paiva, 2007). Therefore, improving bacteriocin production is of great interest for purification, characterization and commercial application of this peptide.

It has been demonstrated that factors affecting the growth of the producer strain, such as media composition, can improve bacteriocin production. Our results indicated that bovicin production by *S. bovis* HC5 was affected by carbon and nitrogen sources.

The highest specific activity of bovicin HC5 was obtained when *S. bovis* was grown in media containing glucose. Several LAB seems to use glucose preferentially for bacteriocin production, and previous studies indicated that *Lactococcus lactis* and *Streptococcus pyogenes* preferred glucose for nisin Z (Matsusaki *et al.,* 1996) and streptococcin A-FF22 production (Jack and Tagg, 1992), respectively. In *Lactococcus lactis*, glucose supports higher specific growth rates, faster substrate consumption and greater product formation, compared to other carbon sources (Even *et al.,* 2001). Recently, Papagianni *et al.* (2007) indicated a direct relationship between nisin production and the rate of glucose consumption by *L. lactis*.

Early work by Russell and Baldwin (1978) demonstrated that ruminal *S. bovis* uses glucose and sucrose preferentially to maltose and cellobiose and the utilization of these sugars was later shown to be regulated by specific phosphotransferase transport systems (PTS) (Martin and Russell, 1987). However, Russell (1990) verified that glucose PTS could not account for the glucose consumption rates of rapidly growing cultures and a low-affinity, facilitated diffusion mechanism was responsible for glucose transport at high substrate concentrations.

These latter results suggest that *S. bovis* can assimilate glucose over a wide range of conditions, which could favor the production of bovicin HC5. However, our results indicated that glucose concentrations greater than 16 g l^{-1} resulted in a decrease in bacterial mass and bovicin activity (Figure 2.1). Papagianni *et al* (2007) observed that batch cultures of *L. lactis* produced less nisin if glucose concentration was above 35 g l^{-1} and this inhibition appeared to be due to a decrease in the rate of glucose uptake. Pattnaik *et al.* (2005) also reported a decrease in bacteriocin production at high glucose concentrations and hypothesized that this inhibition was caused by catabolite repression.

When *S. bovis* HC5 was grown in continuous cultures, the production of bovicin HC5 decreased at high glucose consumption rates and when the culture pH was below 5.4 (Mantovani and Russell, 2003b). In our batch cultures, *S. bovis* HC5 produced lactate even if glucose concentration was as high as 200 g l^{-1}. However, specific growth rate decreased rapidly if glucose concentration was above 40 g l^{-1} and the final culture pH was approximately 4.4 (data not shown).

The peak in cell-free and cell-associated bovicin HC5 activity was detected after glucose consumption and lactate production had stopped and *S. bovis* HC5 cultures reached stationary phase (Figure 2.2). Because Houlihan and Russell (2006b) demonstrated that peptidase activity of *S. bovis* HC5 cultures decreased dramatically at acidic pH values and bovicin HC5 in culture was prevented from being degraded, it appears that the increase in bovicin HC5 activity was due to a greater bacteriocin recovery from *S. bovis* cells. However, bovicin HC5 activity decreased after 24 hours of incubation (Figure 2.2b), even though the culture pH was 4.0. It has been demonstrated that some bovine *S. bovis* strains show competence development and natural genetic transformation (Mercer *et al.,* 1999) and changes in cell surface properties and autolytic activity have also been observed. Therefore, it is conceivable that bacteriocin secretion and the recovery of the cell-associated peptide could vary during growth. Further experiments using antibodies raised against bovicin HC5 will address the binding and the release of bovicin from *S. bovis* HC5 cells at different growth phases.

Among the single nitrogen sources tested in this study, the highest bovicin HC5 specific activity and bacterial cell mass were observed when yeast extract and Trypticase® (0.5 and 1.0 g l^{-1}, respectively) were added together to the basal medium of *S. bovis* HC5. Yeast extract is a common source of amino acids and B-complex vitamins in microbiological media and often stimulates bacterial growth and bacteriocin

production (De Vuyst, 1995; Aasen *et al.,* 2000). Trypticase® and other sources of amino acids seem to balance catabolic and anabolic rates and increase the specific growth rate and growth yield of ruminal *S. bovis* strains (Russell, 1993; Atasoglu *et al.,* 1998).

Previous work by Kim *et al.* (2006) indicated that yeast extract and tryptone were the best nitrogen sources for *Micrococcus* sp. GO5 growth and micrococcin GO5 production, respectively. Furthermore, when these nitrogen sources were used together, bacteriocin production increased. *S. bovis* HC5 produced at least 3 times more bovicin HC5 when the culture medium had yeast extract and Trypticase® combined compared to the use of each source alone (Table 2). Because cell mass production and bacteriocin activity are not readily related, the effect of nitrogen sources on bovicin HC5 production appears to involve multiple physiological responses.

Because some agroindustrial wastes can be used as alternative substrates to sustain bacterial growth and stimulate bacteriocin production, we tested the potential of sugar cane juice and cheese whey to improve bovicin HC5 activity. Cheese whey is a highly polluting, lactose-rich byproduct from cheese industries that has been used for bacteriocin production (Carolissen-Mackay *et al.,* 1997; Alvarez *et al.,* 2006). Sugar cane juice has been commonly used for fuel ethanol fermentation, production of sugar cane spirits and metabolites of industrial interest. However, bacteriocin production on sugar cane juice based media had not been demonstrated.

Our results indicated that *S. bovis* HC5 cultures produced more bovicin HC5 in cheese whey and sugar cane juice than in basal medium, and this observation suggest that bacteriocin production costs could be lowered using these abundant agroindustrial substrates. Because our batch cultures always had more cell-associated than cell-free bacteriocin, the recovery of purified bovicin HC5 could be improved even if the cultures

were grown in these complex, low-cost media. The observation that *S. bovis* had more bovicin HC5 activity when sugar cane juice was used as substrate is probably due to the fact that media containing sugar cane juice provided approximately four times more carbon and energy sources than cheese whey. To our knowledge, this is the first report of the use of sugar cane juice for bacteriocin production.

As previous studies demonstrated that bovicin HC5 has great potential for agricultural and industrial applications, *S. bovis* HC5 might be a useful model to study bacteriocin production. Considering that *S. bovis* HC5 is a versatile LAB that can utilize several carbon and nitrogen sources for bovicin HC5 production, high bacteriocin yields can be attained using alternative substrates. Further studies are being conducted to investigate the effect of physical-chemical factors (e.g. pH, temperature and reduction potential) on bovicin HC5 production and to evaluate its toxicity against animal cell lines.

2.6. Conclusions

S. bovis HC5 was able to grow and produce bovicin HC5 in a wide range of carbon and nitrogen sources and these growth substrates influenced bovicin HC5 yields. These results demonstrate that *S. bovis* HC5 is a versatile LAB that can utilize several carbon and nitrogen sources for bovicin HC5 production. The costs to obtain this bacteriocin can be lowered by using alternative substrates, such as sugar cane juice and cheese whey.

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CHAPTER 3

Effect of growth conditions on bovicin HC5 production by *Streptococcus bovis* HC5

3.1. Abstract

Bacteriocin synthesis is influenced by environmental conditions and the production of bovicin HC5 was investigated under different growth conditions. The conditions that stimulated *S. bovis* HC5 growth was not always related to an increase in bacteriocin production. Results indicated that pH played a major role on bovicin HC5 production. *S. bovis* HC5 grew well in complex and basal media under aerobic and anaerobic conditions, but more bacteriocin amounts were recovered from anaerobic cultures. Lactate production and glucose consumption increased if *S. bovis* HC5 cells were cultivated at pH 7.0 and 45ºC, but bovicin HC5 production was higher at lower pH and temperature values. Cultures under continuous CO_2 flow showed faster growth rates in basal media, but bacteriocin production was higher if *S. bovis* was cultivated in

anaerobic sealed tubes. These results suggest that low pH and anoxic conditions favor the expression of bovicin HC5 in *S. bovis* HC5.

Keywords: bovicin HC5, bacteriocin, pH, environmental conditions, *Streptococcus bovis* HC5

3.2. Introduction

Bacteriocins produced by lactic acid bacteria compose a large and diverse group of ribossomally synthesized, extracellularly released peptides with antibacterial activity against closely related strains (Cleveland *et al.*, 2001). Some peptides have been shown to be effective controlling spoilage and pathogenic microorganisms in foods (Carvalho *et al.*, 2007 ab; Carvalho *et al.*, 2008) or inhibiting human and animal pathogens (Bowe *et al.*, 2006; Coelho *et al.,* 2007).

Bovicin HC5, a bacteriocin produced by *Streptococcus bovis* HC5, has similarity to nisin in its mechanism of action, spectrum of activity, and stability to heat and pH (Mantovani *et al.,* 2002; Houlihan *et al.,* 2004). However, bovicin-sensitive bacteria does not appear to become bacteriocin-resistant as easily as has been shown to nisin (Mantovani *et al.*, 2001; Mantovani and Russell, 2003a; Carvalho *et al.*, 2007ab).

In a previous work, *S. bovis* HC5 was able to produce bovicin HC5 under a wide range of culture conditions (Mantovani and Russell, 2003b), but recent results indicated that carbon and nitrogen sources can affect the amount of cell-free and cell-associated bovicin HC5 (Carvalho *et al.*, 2009). Considering the fact that environmental factors and media composition affect bacteriocin production by lactic acid bacteria (Van Den

Berghe, *et al.,* 2006), we investigated the effect of pH, temperature, aeration and media composition on bovicin HC5 production.

3.3. Materials and methods

3.2.1. Microorganisms and culture media

The bacteriocin producer strain *Streptococcus bovis* HC5 was cultivated anaerobically in basal media containing glucose (16 g l^{-1}), as previously described (Mantovani and Russell, 2003a). The indicator organism, *Alicyclobacillus acidoterrestris* DSMZ 2498, was grown aerobically at 40ºC in *Alicyclobacillus acidoterrestris* medium (AAM), as described by Yamazaki *et al.* (2000).

3.2.2. Bovicin HC5 bioassay

Free bovicin HC5 and cell-associated bacteriocin were determined in the cell-free supernatant and in the acidic extract obtained from cells, respectively. The cell-associated bovicin HC5 was extracted with acidic NaCl, as described by Carvalho *et al.* (2007a). Free bovicin HC5 in the culture supernatant was determined by harvesting the cell-free supernatant from stationary-phase *S. bovis* HC5 cells. Preparations containing bovicin HC5 were serially diluted (2-fold increments) into NaCl solution (100 mmol l^{-1}, pH 2.0) and tested for antimicrobial activity against *A. acidoterrestris* DSMZ 2498 using the agar well diffusion technique described by Hoover and Harlander (1993). Bovicin HC5 activity was estimated from zones of clearing around each well and expressed in terms of arbitrary unit (AU) per mg of cell dry mass or AU ml^{-1}. The

activity of bovicin HC5 was represented as the reciprocal of the highest dilution that still produced a zone of inhibition with at least 10 mm in diameter. The relationship between optical density (600 nm) and cell dry mass for *S. bovis* HC5 was 360 mg cell dry mass liter^{-1} turbidity unit^{-1} as previously described (Carvalho *et al.*, 2009).

3.3.3. Effect of aeration and media composition on bovicin HC5 production

S. bovis HC5 was grown in BHI (Becton, Sparks, MD, USA), MRS (Himedia, Mumbai, India), M17 (Sigma, Buchs, Switzerland) or basal media (Mantovani and Russell *et al.*, 2003). Media pH was always adjusted to pH 6.5 with NaOH (1 mol^{-1}) and incubations were carried out under aerobic and anaerobic conditions. The cultures were maintained at 39ºC and the growth was monitored spectrophotometrically (OD_{600nm} - Spectronic 20D+, Thermal Electron, Madison, WI) at time intervals for 24 h. The cell-free supernatants and the acidic bacteriocin extracts obtained after 16 h of growth in each medium were tested for bovicin HC5 activity by well diffusion assay. The specific growth rate and final pH of the cultures were also determined.

S. bovis HC5 was also grown in basal media under three different conditions: 1) under continuous CO_2 flux (CCF), 2) in sealed anaerobic tubes (SAT) and 3) under aerobic conditions. The changes in optical density and bacteriocin production were monitored during *S. bovis* HC5 growth as described above.

3.3.4. Production of bovicin HC5 at different pH values and incubation temperatures

To assess the effect of initial pH on bovicin HC5 production, *S. bovis* HC5 was cultivated anaerobically in basal media lacking Na_2CO_3. The media pH was adjusted to values ranging from 4.5 to 7.0, using HCl or NaOH at 1 mol l^{-1}. Each tube was inoculated with 3% (v/v) of an 18 h-old culture of *S. bovis* HC5 and incubated at 39ºC for 16 h. Growth was monitored at time intervals by determining the changes in OD_{600nm} in a Spectronic 20D+. In another experiment, the culture pH was maintained at constant values in the range that *S. bovis* was able to grow (5.5 to 7.0) using a pH controller (Model 5656-00, Cole Parmer, Illinois, USA). *S. bovis* HC5 was grown in 500 ml fleaker beaker flasks (Corning) that were continuously purged with O_2-free carbon dioxide and after incubation times of 16 and 24 hours, samples of 50 ml were withdrawn and OD value and bovicin HC5 activity was determined in acidic NaCl extract and in culture supernatant as described above. Glucose and fermentation acids in cell-free supernatant were analyzed by high performance liquid chromatography (HPLC, Bio-Rad HPX-87H organic acid column). The sample size was 20 µl, the eluant was 0.005 mol l^{-1} H_2SO_4, the flow rate was 0.7 ml min^{-1} and the column temperature was 60ºC. Control treatments without pH control were also performed.

The effect of temperature on *S. bovis* HC5 growth and bovicin production was determined incubating cultures of *S. bovis* HC5 anaerobically on basal media for 16 h at temperatures of 25, 30, 36, 39 and 45ºC. After the incubation time, final pH and bovicin HC5 specific activity, expressed as AU per mg dry cell mass, were determined for all the pH and temperature conditions described above.

3.3.5. Statistics

All experiments were carried out in duplicate and repeated twice. When error bars are given in the figures, they refer to the standard deviation of the mean.

3.4. Results

3.4.1. Effect of different growth media on *S. bovis* HC5 growth and bacteriocin production

When *S. bovis* HC5 was grown in different media, the specific growth rate and biomass production were always greater at anaerobic conditions (Table 3.1). Bovicin HC5 was detected in the cell-free supernatant and in the acidic NaCl extracts obtained for all the conditions tested (Table 3.1). However, biomass production and higher specific growth rate were not always related to increased bovicin HC5 production (Table 3.1).

When MRS, M17 or BHI broth were used, the lower specific growth rates attained at aerobic conditions was related to a greater bovicin HC5 activity, particularly in the cell-free supernatant (Table 3.1). However, the reverse was observed to basal media. In this latter treatment, higher cell densities and specific growth rates were related with greater bovicin HC5 production in the cell extract (Table 3.1). The greatest bovicin HC5 activity was observed in basal media under anaerobic conditions (Table 3.1). Because complex media seemed to reduce bacteriocin yield, basal media was chosen to be used in subsequent experiments.

Table 3.1. *S. bovis* HC5 growth and bovicin HC5 production in different culture media

Media	Growth condition	Final pH*	μ (h^{-1})	Final OD	Bovicin HC5 specific activity (AU ml^{-1} mg dry cell $mass^{-1}$)	
					Supernatant	Extract
MRS	Aerobiose	4.53	0.55	2.01	889	444
	Anaerobiose	4.19	0.73	4.01	83	28
M17	Aerobiose	4.33	0.41	2.02	888	333
	Anaerobiose	4.45	0.75	3.75	178	60
BHI	Aerobiose	4.53	0.59	1.38	160	80
	Anaerobiose	5.27	0.99	3.30	133	67
Basal	Aerobiose	4.60	0.20	2.05	432	1730
media	Anaerobiose	4.20	1.10	3.20	914	5485

* Initial pH was 6.5

3.4.2. Effect of pH and temperature on bovicin HC5 production

Batch culture experiments indicated that *S. bovis* HC5 could grow anaerobically in basal medium even if the initial pH was as low as 5.5, but the rate of growth and final optical density increased at higher pH values (Table 3.2). Cell-associated bovicin HC5 specific activity was highest (4923 AU ml^{-1} mg dry cell $mass^{-1}$) in basal media at pH 6.5, but it reduced approximately 67%, 70% and 90% if the initial pH was 5.5, 6.0 and 7.0, respectively (Table 3.2).

Table 3.2. Effect of medium pH on growth and production levels of bovicin HC5 by *S. bovis* HC5

Initial pH	Final pH	Final OD	μ (h^{-1})	Bovicin HC5 specific activity (AU $ml^{-1}mg^{-}$ dry cell $mass^{-1}$)	
				Supernatant	Extract
4.5	4.50	0.136	NG	0	0
5.0	5.00	0.130	NG	0	0
5.5	4.00	0.540	0.45	400	1600
6.0	3.80	1.255	0.82	355	1422
6.5	3.85	2.900	0.95	307	4923
7.0	3.90	3.790	1.15	235	470

NG = No growth

When *S. bovis* HC5 was grown in basal medium with pH control, bovicin HC5 specific activity increased with the reduction in the media pH (Table 3.3). At pH 5.5 higher bovicin HC5 activity was observed, as in the cell extract as in the culture supernatant, independently of incubation time (Table 3.3). The bovicin HC5 activity in the cell extract at this pH value was approximately 100 times higher than control treatments after 24 h of incubation (Table 3.3). At pH 6.0 and 6.5, bovicin HC5 specific activity was lower than at pH 5.5, but approximately 70% higher than control (Table 3.3). Bovicin HC5 activity was not detected in the culture supernatant when the pH was maintained at 6.5 and 7.0 (Table 3.3).

Control treatment showed similar levels of bovicin HC5 activity in the acidic extract at 16 and 24 hours of incubation, as also observed to pH 6.0 and 6.5 (Table 3.3). However, at pH 5.5 the bacteriocin activity increased in more than 50% after 24 hours. No bacteriocin was detected at pH 7.0 at 24 h. Biomass production by *S. bovis* HC5 after 24 h of incubation at pH values ranging from 5.5 to 6.5 averaged 1.35 mg ml^{-1}

(Table 3.3). At pH 7.0 and in control treatments, lower values of microbial biomass were obtained (Table 3.3).

Table 3.3. Effect of controlled pH on bovicin HC5 production by *S. bovis* HC5

pH	Time	Biomass (mg ml^{-1})	Bovicin HC5 specific activity (AU ml^{-1}mg dry cell $mass^{-1}$)	
			Supernatant	Extract
5.5	16 h	1.17	3096	23032
	24 h	1.37	66064	518516
6.0	16 h	1.2	254	14380
	24 h	1.38	3047	14380
6.5	16 h	1.29	0	14576
	24 h	1.39	0	11288
7.0	16 h	1.04	0	1207
	24 h	0.96	0	0
Control	16 h	0.9	1536	4960
	24 h	1.03	604	4490

When the culture supernatant was analyzed by HPLC, lactate was the only metabolic product of glucose fermentation (results not shown). Lactate concentration and glucose consumption increased at higher pH values (Figure 3.1).

When *S. bovis* HC5 was cultivated in basal media (pH 6.5) at different temperatures, growth occurred at temperatures ranging from 30ºC to 45ºC (Figure 3.2a). Culture pH after 16 h of incubation was approximately 4.0 and typical optical densities varied from 2.0 at 30ºC through 39ºC to 2.6 at 45ºC (Figure 3.2a). Approximate levels of 13000 AU ml^{-1} mg cell dry $mass^{-1}$ of bovicin HC5 were obtained when *S. bovis* HC5

was incubated at 30ºC, 36ºC or 39ºC. However, a decrease of approximately 20% was observed at 45ºC (Figure 3.2b).

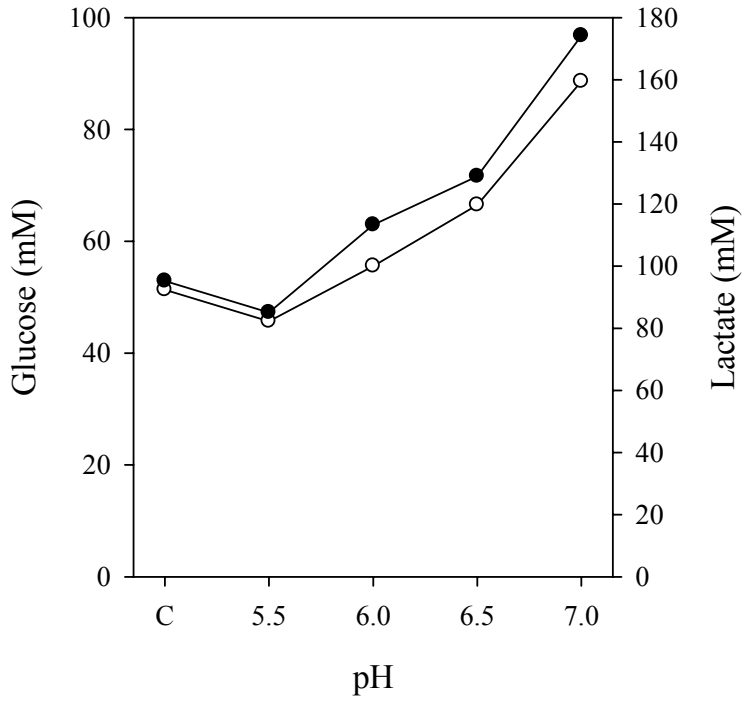

Figure 3.1. The consumption of glucose (open circles) and production of lactate (closed circles) by *S. bovis* HC5 grown at different pH values. *S. bovis* was inoculated into basal media added with glucose at 16 g l^{-1} and the media pH was maintained at 5.5, 6.0, 6.5 and 7.0. After 24 hours of incubation samples were taken and fermentation products and residual glucose were analyzed in culture supernatants. The control treatments (C) without pH control are also shown.

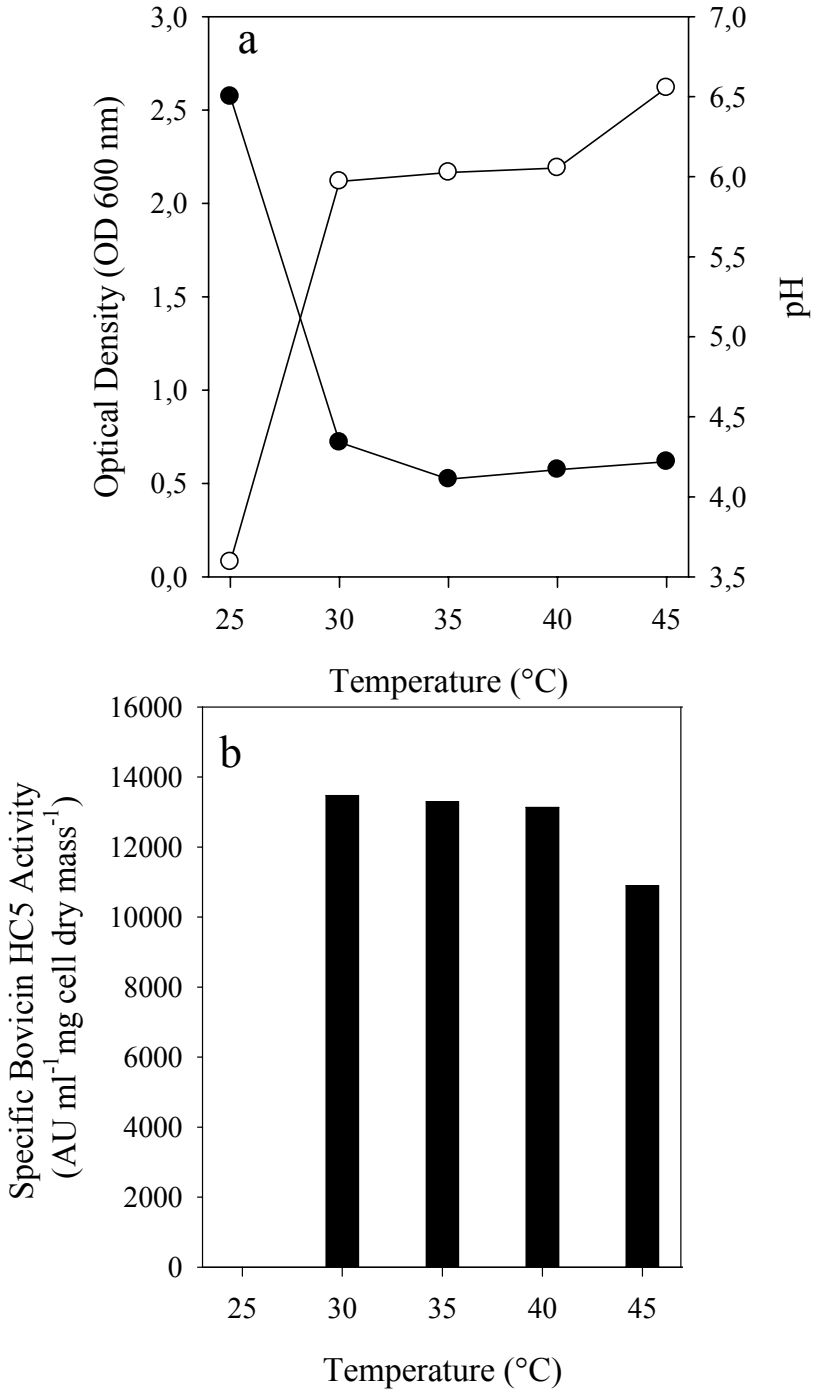

Figure 3.2. Effect of temperature on *S. bovis* HC5 growth and bovicin HC5 production. *S. bovis* HC5 was cultivated anaerobically in basal media (pH 6.5) incubated for 24 h at temperatures ranging from 25ºC to 45ºC. (a) The final pH (closed circles) and the $OD_{600\ nm}$ (open circles) were measured. The specific activity of bovicin HC5 against *A. acidoterrestris* DSMZ 2498 is indicated in (b) for cell-free extract.

3.4.3. Growth and bacteriocin production by *S. bovis* in basal media at different aeration conditions

When *S. bovis* HC5 was cultivated in basal media (pH 6.5) under conditions of continuous CO_2 flux (CCF), sealed anaerobic tubes (SAT) and aerobic condition, the bacteriocin producer strain grew under all conditions tested with a lag phase period of 2 hours (Figure 3.3). However, the specific growth rate was greater in CCF and maximal biomass production was observed in SAT (Figure 3.3).

Bovicin HC5 was monitored during *S. bovis* HC5 growth and was detected at all conditions (Figure 3.4). Bacteriocin production was observed after 2 hours of aerobic growth (160 AU ml^{-1}) or 4 h of anaerobic growth (320 AU ml^{-1}). The increase in bovicin HC5 activity was faster under CCF condition (Figure 3.4). Maximal bacteriocin activity (AU ml^{-1}) was observed in the acidic cell-extract at 24 h of growth, and the activity was higher when *S. bovis* was cultivated in sealed tubes (10240 AU ml^{-1}, Figure 3.4).

Bovicin HC5 activity reduced about 33% and 50% after 36 hours of incubation in continuous CO_2 flux and sealed tubes, respectively. Under aerobic conditions this decrease in bacteriocin activity was not observed at 24 h of incubation (Figure 3.4). However, after 48 h, reduction in bovicin HC5 activity was observed for all conditions tested, being more pronounced to cultures growing at SAT (Figure 3.4).

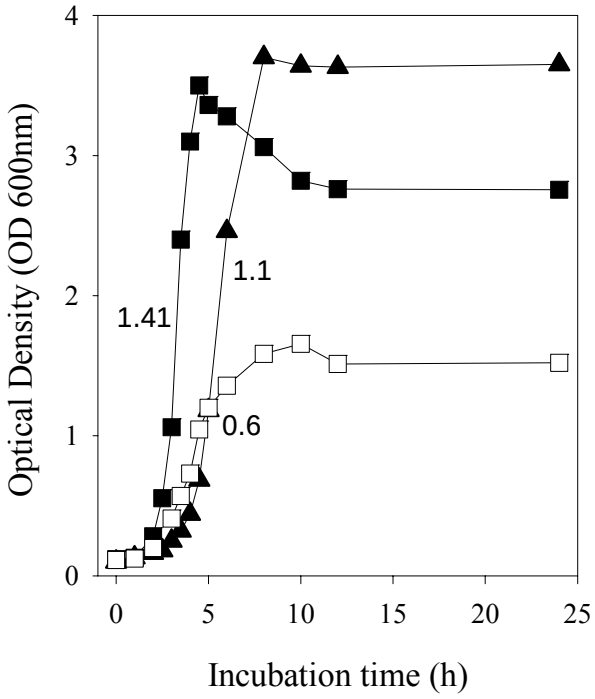

Figure 3.3. The effect of growth atmosphere on *S. bovis* HC5 growth. *S. bovis* HC5 was incubated under aerobic condition (open squares), continuous CO_2 flow (closed squares) and in sealed anaerobic tubes (closed triangles). The cultures were maintained at 39ºC and the growth was monitored by changes in optical density ($OD_{600\ nm}$). The estimated specific growth rates for *S. bovis* HC5 are indicated for each growth condition.

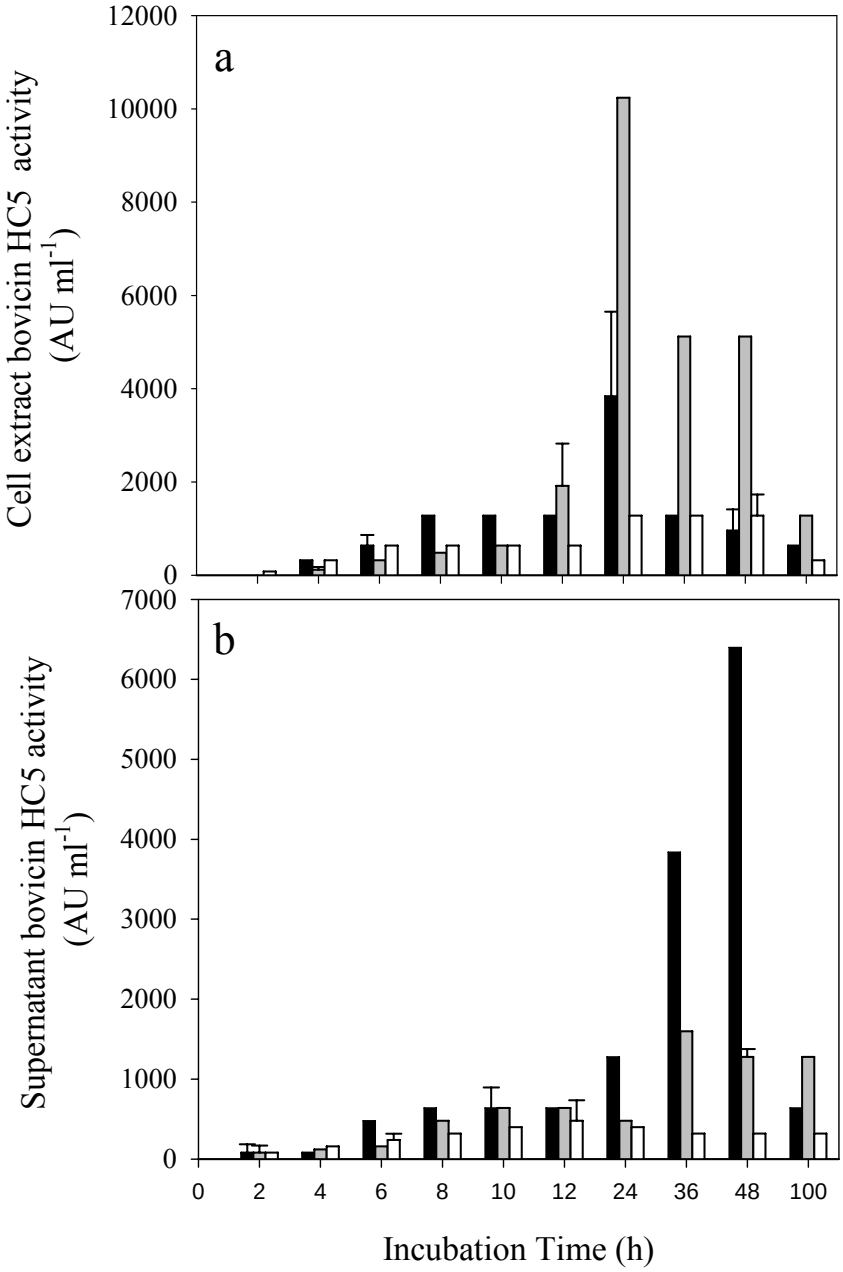

Figure 3.4. Production of bovicin HC5 under aerobic conditions (white bars), continuous CO_2 flow (black bars) and sealed anaerobic tubes (gray bars). At each time interval, samples were taken and bovicin HC5 activity (AU ml^{-1}) in the cell extract (a) and in the culture supernatant (b) were determined using *A. acidoterrestris* DSMZ 2498 as the indicator organism.

3.5. Discussion

Environmental factors, such as nutrient availability, pH, temperature and atmosphere have great influence on bacteriocin production by lactic acid bacteria (Leroy and De Vuyst, 1999; Mataragas *et al.,* 2003). Due to the fastidious character of these microorganisms, high levels of bacteriocin production are generally achieved in defined or complex media (Kim *et al.*, 2006). Strains of *Pediococcus acidilactici* (Anastasiadou *et al.,* 2008), *Lactococcus lactis* (De Vuyst *et al.,* 1996) and *Micrococcus* sp. GO5 (Kim *et al.,* 2006) appears to grow better and produce more bacteriocin in complex media.

To be commercially attractive, bacteriocins should be obtained at high yields. *Streptococcus bovis* HC5 is a lactic acid bacterium that has few nutritional requirements, being able to use ammonia as the sole nitrogen source (Wolin *et al.,* 1959). Our results show that *S. bovis* HC5 grows better and produces higher levels of bovicin HC5 in basal media than in the complex media. Previous results also showed that this bacterium was able to grow and produce high levels of bovicin HC5 on media containing sugar cane juice or cheese whey as carbon sources (Carvalho *et al.*, 2009).

S. bovis HC5 was able to grow and produce bovicin HC5 in basal media at pH values ranging from 5.5 to 7.0 and temperatures from 30°C to 45ºC. High growth rates and biomass production were obtained when *S. bovis* HC5 was cultivated at pH 7.0 and incubated at 45ºC. However, maximal bovicin HC5 production was detected at suboptimal initial pH (6.5) and at temperatures ranging from 30°C to 39ºC. Cultures in which the culture pH was maintained at 5.5 also had high levels of bovicin HC5. The fact that the conditions for optimal bacteriocin production did not coincide with the optimum growth conditions for *S. bovis* HC5 was also reported for other bacteriocin-

producing strains (Aasen *et al.,* 2000; Mataragas *et al.,* 2003; Drosinos *et al.,* 2006). Considering that bacteriocin production by lactic acid bacteria is viewed as a competitive strategy, under unfavorable growth conditions bacteriocin production could be advantageous to compete for limited resources.

Temperature and pH not only influenced the growth of the producer strain but also can affect the stability of the peptide by interfering in post-translational modification, aggregation, absorption to cells, proteolysis and the activity of the bacteriocin (Cheigh *et al.,* 2002; Drosinos *et al.*, 2006). Our results indicated that pH values below and above 6.5 affected negatively bovicin HC5 production. Houlihan *et al.* (2004) did not studied the effect of pH on bacteriocin production by *S. bovis* HC5, but showed that bovicin HC5 activity was highly pH dependent and enhanced at acidic conditions. Because we always used acidic saline solution to access bovicin HC5 activity, the difference in bacteriocin activity is attributed to bacteriocin production rather than activity. Based on these results, it appears that pH could influence bovicin HC5 production.

When *S. bovis* HC5 grew at constant pH, higher bovicin HC5 activity was detected at pH 5.5. Similar results have been reported by Mantovani and Russell (2003b) who reported a 2-fold increase in the antibacterial activity of *S. bovis* HC5 grown in continuous culture when the pH was decreased from 6.7 to 5.4. Previous work indicated that the synthesis of lacticin 481, nisin and pediocin is activated at transcriptional level by acidic pH (Guerra and Pastrana, 2003; Hindré *et al.,* 2004). A lack of antimicrobial activity was observed in culture supernatants when *S. bovis* HC5 was maintained at pH 6.5 and 7.0. These results agree with the work of Houlihan and Russell (2006) whom showed that bovicin HC5 remain cell-associated at neutral pH. In

addition, peptidase activity also increases at this pH value, probably reducing the bovicin HC5 activity (Houlihan and Russell, 2006).

Under these conditions, glucose consumption by *S. bovis* HC5 followed the variation in pH. Glucose uptake by *S. bovis* HC5 is regulated by specific phosphotransferase transport systems (PTS) (Martin and Russell 1987) and a facilitated diffusion mechanism also exists for glucose transport at high substrate concentrations (Russell, 1990). pH could interfere in the activity of proteins involved in these mechanisms and Moore and Marti (1991) showed that the optimum activity of the phosphotransferase system of *S. bovis* JB1 occurred at pH 7.2. Therefore, at high pH values, the uptake of glucose into cells is expected to increase.

Higher glucose consumption and lactate production rates were observed at pH 7.0. However, less biomass and bacteriocin were produced at this condition. Previous work demonstrated that *S. bovis* could spill energy when glucose is added in excess to the culture media (Russell and Strobel, 1990). Bond and Russell (1996) also reported a correlation between energy spilling and lactate production in *S. bovis*. Further studies indicated that fructose 1,6-diphosphate is involved in the ATPase-mediated energy-spilling reaction. Our results suggest that pH could also be an important factor to energy-spilling reactions in *S. bovis* HC5 and this mechanism could also influence bacteriocin production. If energy-spilling reactions are occurring, less ATP is made available for bacteriocin production.

Leroy and De Vuyst (1999) and Drosinos *et al.* (2006) found that when temperature increased at certain level, bacteriocin activity decreased. The authors attributed this to a higher protease activity or cell-bacteriocin or bacteriocin–bacteriocin interaction. *S. bovis* strains are known to produce peptidases (Russell and Robinson,

1984), therefore the lower activity observed at 45ºC could be attributed to a decrease in bovicin HC5 activity, due to higher peptidase activity.

Lactic acid bacteria grow under microaerophilic to strictly anaerobic conditions (Klein *et al.*, 1998). Although production of many bacteriocins produced by LAB has been studied under anaerobic conditions, to some bacteriocins, like nisin (Cabo *et al.*, 2001), an oxygen-enriched atmosphere enhanced production. *S. bovis* HC5 was also able to grow and produce bacteriocin at aerobic condition. However, the specific growth rate and bovicin HC5 levels at this condition were much lower. Aeration should affect bacteriocin production as the oxygen tolerance by lactic acid bacteria is associated to different metabolic pathways that could affect bacteriocin production (Neysens and De Vuyst, 2005). *S. bovis* HC5 is a homolactic bacterium, but at some conditions, it can switch its fermentation to acetate, formate and ethanol to obtain more ATP (Russell and Baldwin, 1979). An aerobic atmosphere can modify the metabolic products and, consequently, negatively interfere in bacteriocin production. When the metabolic products of *S. bovis* HC5 grown aerobically was analyzed only lactate was detected (results not shown).

Neysens and De Vuyst (2005) showed that amylovorin titers were higher when the producer strain was maintained at anaerobic condition, and highest under carbon dioxide flow rates. In the case of *S. bovis* HC5, the effect of a continuous CO_2 supply on the growth media was mainly reflected by a high specific growth rate and a decrease in bovicin HC5 production compared to cultivation in anaerobic sealed tubes. These results indicated that the CO_2 level could be an important factor to enhance bovicin HC5 production. Because we did not measure the CO_2 concentration in the sealed tubes or in the flow line, it appears that low CO_2 concentration at anaerobic atmosphere could favor bovicin HC5 production.

3.6. Conclusions

These results indicate that several environmental factors affect cell yield and bovicin HC5 production in *S. bovis* HC5. However, culture pH and atmosphere were the factors that showed a more pronounced effect on bovicin HC5 production. Conditions that favored *S. bovis* HC5 growth were not always related to a greater bacteriocin production or secretion. Although the experiments were not conducted at molecular level, they contributed for a better understanding of the influence of environmental factors on bovicin HC5 production. However, to clarify their effect on the regulation of bovicin HC5 production, real-time PCR of the structural genes encoding production of this bacteriocin could be done. The structural gene of this bacteriocin was not sequenced yet, but studies to obtain the nucleotide sequence of this bacteriocin are being conducted.

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CHAPTER 4

Effect of pH and growth rate on bovicin HC5 production by *Streptococcus bovis* HC5

4.1. Abstract

The metabolic pattern of *S. bovis* HC5 can be changed from homolatic to mixe-acid fermentation by growing the cells at lower specific growth rates and at neutral pH. This work aimed to investigate the effect of continuous culture dilution rates and extracellular pH on growth and bovicin HC5 production by *S. bovis* HC5. When *S. bovis* HC5 was cultivated at dilutions rates ranging from 0.07 to 1.20 h^{-1} at uncontrolled pH conditions, glucose was metabolized mainly to lactic acid and bacteriocin production was higher at lower dilution rates. Under these conditions, extracellular pH was approximately 5 and glucose was not completely consumed. If media pH was maintained at 7.5, mixed-acid fermentation was observed for cultures grown at dilution rates below 1.20 h^{-1}. Bacteriocin production increased at higher dilution rates, but the levels were at least 10 times lower when compared to uncontrolled pH conditions. Residual glucose was only detected in the fermentator at dilution rates above 0.60 h^{-1}.

These results indicate that both culture pH and growth rate control *S. bovis* HC5 metabolism and bovicin HC5 production is not always growth associated. Our results showed that pH and growth rate affect bovicin HC5 production, but the physiological mechanisms involved in this effect needs to be clarified.

Key words: *Streptococcus bovis* HC5, bovicin HC5, bacteriocin production, metabolism change, continuous culture

4.2. Introduction

Streptococcus bovis HC5 converts sugars via the glycolytic pathway to lactate, generating energy mainly through substrate-level phosphorylation (Russell, 1990). It has been suggested that during homolactic fermentation, regulation of the carbon flux is associated with high levels of fructose 1,6-disphosphate (FDP) and the subsequent activation of lactate dehydrogenase and pyruvate kinase (PK), thus directing the metabolic flow towards the production of lactate (Cocaign-Bousquet *et al.,* 1996; Garrigues *et al.,* 1997; Neves *et al.*, 2005).

Wolin (1964) noted that the lactate dehydrogenase of *S. bovis* required FDP as an allosteric activator and this glycolytic intermediate also appears to regulate the activity of the F_1F_o ATPase (Bond and Russell, 1996). FDP has also been reported as a major allosteric effector of the catabolite control protein A (CcpA) and PK (Neves *et al.*, 2005)

A metabolic shift in *S. bovis* from homolactic to mixed acid fermentation (acetate, ethanol and formate production) can be obtained by growing the cells at lower specific growth rates and at pH values near to neutrality (Russell, 1991; Asanuma and

Hino, 2000). It is postulated that at low fermentation rate the cells will have little FDP, lactate dehydrogenase will not be activated, and glucose will also be converted to acetate, formate, and ethanol. At these conditions, the levels of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate are also low and the inhibition of pyruvate formate lyase is relieved (Garrigues *et al.,* 1997). The activity of the pyruvate formate lyase is also affected by pH, being activated at near neutral pH values (Asanuma and Hino, 2000).

Previous studies have shown that manipulation of pH alone is not sufficient to change *S. bovis* HC5 fermentation pattern. Considering that different metabolic pathways yield different products (Neysens and De Vuyst, 2005), both cell mass and bacteriocin production could be affected. The following experiments were designed to induce changes in the fermentation pattern of *S. bovis* HC5 and to investigate the effect of continuous culture dilution rates and extracellular pH on growth and bovicin HC5 production by *S. bovis* HC5.

4.3. Materials and Methods

4.3.1. Microorganisms and growth conditions

Streptococcus bovis HC5 was grown anaerobically in a basal media (39°C), as previously described (Mantovani and Russell, 2003). The basal medium contained (per liter): 292 mg K_2HPO_4, 292 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4.7H_2O$, 64 mg $CaCl_2.2H_2O$, 500 mg cysteine hydrochloride, 1 g Trypticase®, 0.5 g yeast extract, 4 g Na_2CO_3 and 16 g glucose. The medium was prepared anaerobically under an O_2-free carbon dioxide flux and the final pH was adjusted to 6.5 with NaOH (1

mol l^{-1}). The indicator strain, *Alicyclobacillus acidoterrestris* DSMZ 2498, was grown at 42ºC in *Alicyclobacillus acidoterrestris* medium (AAM), as described by Yamazaki *et al.* (2000).

4.3.2. Bovicin HC5 activity

Cell-free and cell-associated bovicin HC5 were determined in the culture supernatant and in acidic NaCl extracts obtained from cell pellets, respectively. *S. bovis* HC5 cells (50 ml culture volume) were harvested by centrifugation (9000 x *g*, 4ºC, 15 min) and the culture supernatant was used to assay the activity of cell-free bovicin HC5 (S). The cell pellet was re-suspended in a volume of acidic NaCl solution (100 mmol l^{-1}, pH 2.0) that corresponded to 5% of the initial culture volume. Cell suspensions were incubated under agitation (approximately 150 rpm) for 2 h at room temperature, followed by centrifugation (9000 *x g*, 4ºC, 15 min) and determination of bacteriocin activity in the cell-free extract (E-2h). Cells underwent a second extraction by re-suspending the pellet in the same volume of acidic NaCl and incubating the cells at 100°C for 20 minutes. The cells were centrifuged again and bovicin HC5 activity in the cell-free extract (E-100°C) was determined.

Preparations containing bovicin HC5 were serially diluted (2-fold increments) into NaCl solution (100 mM, pH 2.0) and tested for antimicrobial activity against *A. acidoterrestris* DSMZ 2498 using the agar well diffusion technique described by Hoover and Harlander (1993). One arbitrary unit (AU, expressed per cell dry mass) was defined as the reciprocal of the highest dilution that showed a zone of inhibition with radius of at least 5 mm.

4.3.3. Continuous culture experiments

Fermentation flasks (500 ml; Bellco, USA) containing anaerobic basal media (300 ml) added with 16 g l^{-1} of glucose were continuously purged with O_2-free CO_2. *S. bovis* HC5 cells were inoculated (3% inoculum, v/v) and incubated at 39°C under agitation (150 rpm). The pH was maintained constant at 7.5 with NaOH (4 M) by using a pH controller (Model 5656-00, Cole Parmer, Illinois, USA). Alternatively, the fermentation flasks were incubated under the same conditions without pH control. The dilution rate was maintained at 0.07, 0.15, 0.30, 0.60 or 1.20 h^{-1} using a Masterflex® microprocessor pump (Model 7524-10, Cole Parmer, Illinois, USA). After the cultures reached the steady state for each dilution rate, samples of 50 ml were harvested. The steady-state condition for each dilution rate was monitored by determining the optical densities of the cultures at 600 nm using a microplate reader (TP reader, Thermo Plate, Waltham, MA) and samples were not taken until at least four culture vessel volumes of medium had passed through the continuous culture vessel (or a 98% turnover). The activity of cell-free and cell-associated bovicin HC5 was determined for each dilution rate as described above.

4.3.4. Other analyzes

Cell dry mass for *S. bovis* HC5 was determined as previously described (Carvalho *et al.*, 2009). Residual glucose and organic acids in cell-free supernatants were analyzed by high performance liquid chromatography (HPLC, HPX-87H column, Bio-Rad, Hercules, CA). The sample size was 20 µl, the eluant was 0.005 mol l^{-1} H_2SO_4, the flow rate was 0.7 ml min^{-1} and the column temperature was 60ºC.

4.3.5. Statistical analysis

All treatments were carried out in at least two independent experiments and reported data represent arithmetic mean values. When error bars are given in the figures, they refer to the standard deviation of the mean.

4.4. Results

4.4.1. Growth and bacteriocin production by *S. bovis* HC5 in continuous culture without pH control

When *S. bovis* HC5 was grown in continuous culture without pH control biomass production increased with dilution rates up to 0.30 h^{-1}, but a rapid decrease in biomass production was observed at faster dilution rates (Figure 4.1a). The average pH for cultures at dilution rates of 0.07 h^{-1} and 0.15 h^{-1} was 5.0. Culture pH increased at faster dilution rates, reaching values of 6.4 at rates of 1.20 h^{-1} (Figure 4.1a). As expected, residual glucose also increased with dilution rate (Figure 4.1b).

Under conditions of uncontrolled pH lactate was the only organic acid produced from glucose, regardless of the dilution rate. Lactate production reduced as the dilution rate increased (Figure 4.1b). For dilution rates ranging from 0.07 h^{-1} to 0.60 h^{-1}, lactate concentration decreased only 10 mM (90 to 80 mM), but less than 40 mM was produced at dilution rates of 1.20 h^{-1} (Figure 4.1b).

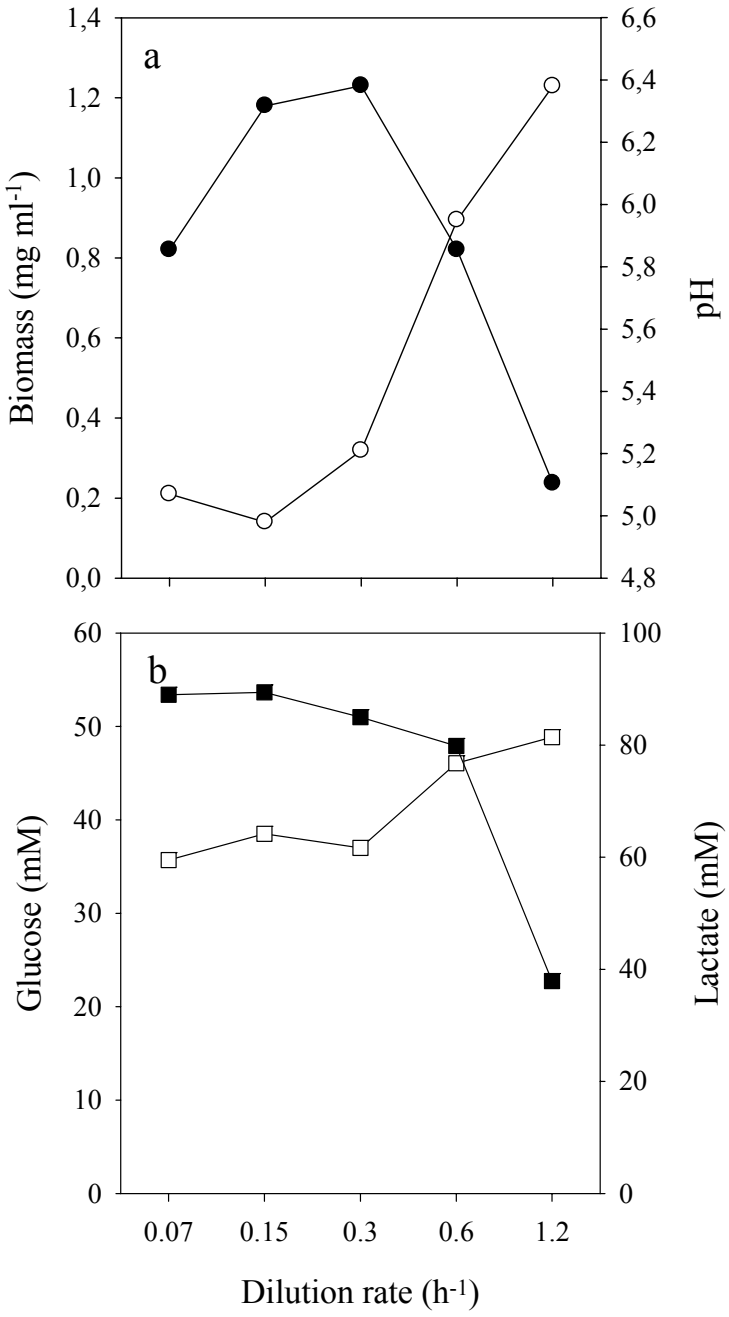

Figure 4.1. Effect of dilution rates on growth (a) and glucose fermentation (b) by *S. bovis* HC5. Cells were cultivated in basal media added with glucose at 16g l^{-1} and the dilution rate varied from 0.07 h^{-1} to 1.20 h^{-1}. After the culture reached steady-state, samples were withdrawn and analyzed for biomass production (closed circles), culture pH (open circles), residual glucose (open squares) and lactate production (closed squares).

The specific activity of cell-free and cell-associated bovicin HC5 was higher at lower dilution rates (Figure 4.2). Bovicin HC5 was extracted twice from the cells first at room temperature (E-2h) and then at high temperature (E-100°C), and even this second extraction yielded high levels of bovicin HC5 at some dilution rates (Figure 4.2). The acidic NaCl extracts obtained at room temperature appeared to be more effective against *A. acidorerrestris* (Figure 4.2). Very little cell-free bovicin HC5 activity was found in the supernatants of cultures maintained at different dilution rates.

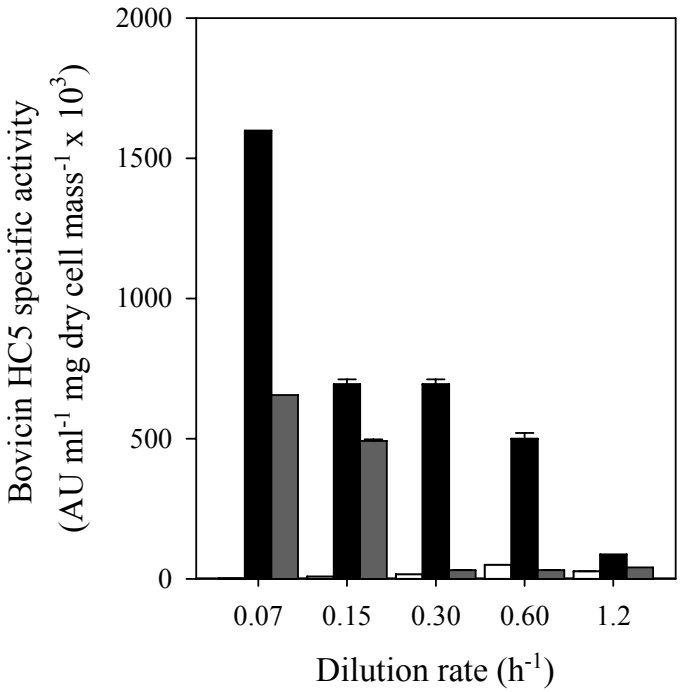

Figure 4.2. Effect of dilution rate on bovicin HC5 production by *S. bovis* HC5 using *A. acidoterrestris* as indicator strain. Cells were grown in basal media added with glucose at 16 g l^{-1} and the dilution rate varied from 0.07 to 1.20 h^{-1}. After cultures reached steady state, samples were withdrawn and bovicin HC5 activity was determined by the agar well diffusion assay in culture supernatant (white bars), cell extracts obtained by acidic NaCl treatment of cells for two hours (black bars) and at 100 °C (gray bars).

4.4.2. Growth and bacteriocin production by *S. bovis* HC5 in continuous culture at controlled pH

When *S. bovis* HC5 was maintained under controlled pH (7.5) in continuous culture, a change in metabolic pattern was observed. Lactate increased compared to cultures under uncontrolled pH conditions and acetate, formate and ethanol were produced (Table 4.1). The concentration of organic acids decreased at higher dilution rates and lactate was always at greater concentration compared to other acids (Table 4.1). At dilution rate of 1.20 h^{-1} only lactate was detected in the culture supernatant (Table 4.1).

Biomass production increased in dilution rates ranging from 0.07 h^{-1} to 0.30 h^{-1} and glucose was completely consumed (Figure 4.3). If the dilution rate was increased to 0.60 h^{-1} or 1.20 h^{-1}, biomass production decreased abruptly and glucose was no longer completely utilized (Figure 4.3).

Table 4.1. Fermentation products of *S. bovis* HC5 cultures maintained in continuous culture under conditions of controlled pH (7.5) at different growth rates

Dilution rate (h^{-1})	Fermentation products (mM)			
	Lactate	**Formate**	**Acetate**	**Ethanol**
0.07	117.88	51.90	23.19	27.39
0.15	121.63	35.72	16.72	41.02
0.30	137.81	24.07	9.65	25.00
0.60	72.74	3.15	1.20	11.16
1.20	59.34	-*	-*	-*

-* The organic acid was not detected under these conditions.

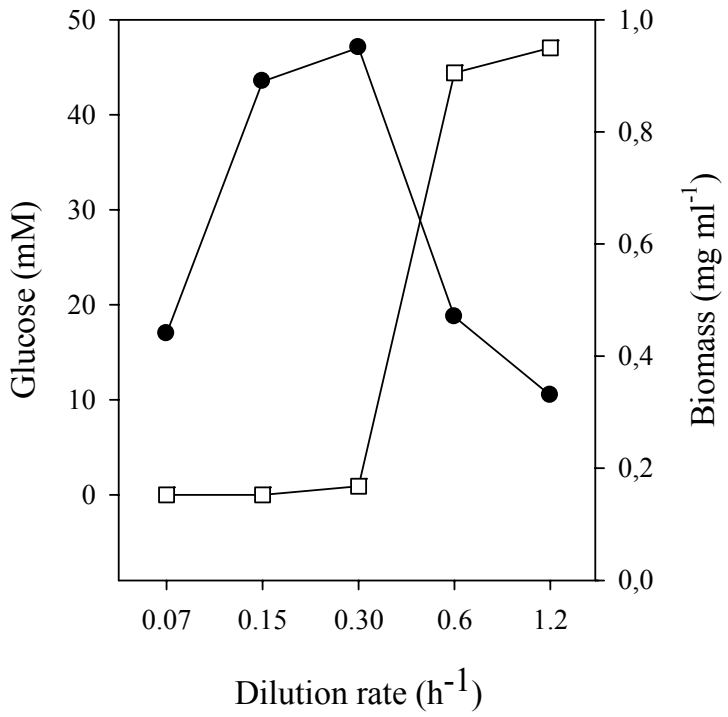

Figure 4.3. The effect of dilution rate on biomass production (closed circles) and glucose utilization (open squares) by continuous cultures of *S. bovis* HC5. Cells were grown anaerobically in basal media added with 16 g l^{-1} glucose. The pH was maintained at 7.5 during incubation using NaOH (4 M). After the culture reached steady state, samples were withdrawn and biomass and residual glucose were determined.

Bovicin HC5 activity was detected at all dilution rates tested (Figure 4.4). Greater inhibition was observed for extracts obtained at 100°C, especially at the dilution rate of 1.20 h^{-1} (93.090 AU ml^{-1} mg dry cell $mass^{-1}$; Figure 4.4). Cell-free bovicin HC5 was detected in culture supernatants of all dilution rates (Figure 4.4).

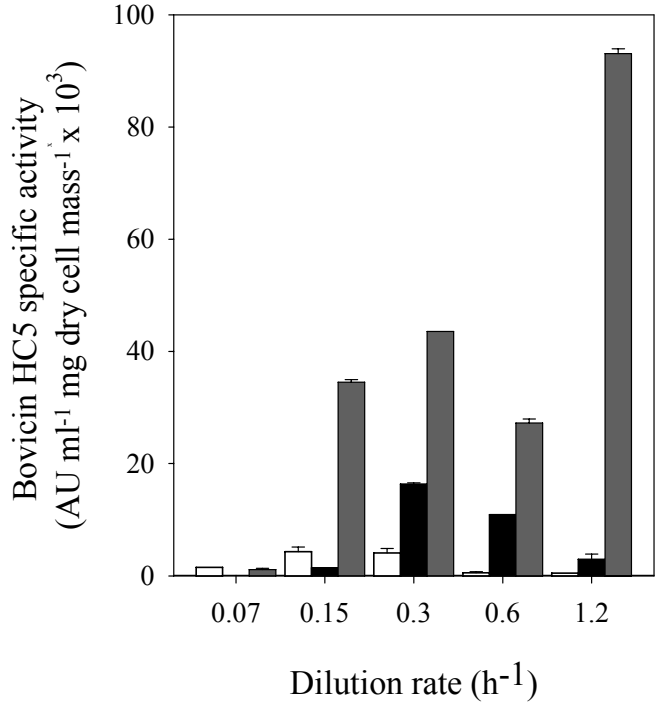

Figure 4.4. Effect of continuous culture dilution rate on bovicin HC5 production by *S. bovis* HC5 using *A. acidoterrestris* as indicator strain. Cells were grown in basal media added with 16 g l^{-1} glucose and with pH maintained at 7.5. After cultures reached steady state, samples were harvested and bovicin HC5 activity was assayed in culture supernatants (white bars) or in cell extracts obtained from acidic NaCl treatment of the cells for two hours (black bars) or at 100°C (gray bars).

4.5. Discussion

Continuous cultures of *S. bovis* HC5 without control of pH metabolized glucose exclusively to lactate. The final culture pH under these conditions was below 5.0, a pH that is known to inhibit the activity of pyruvate formate lyase (PFL). Asanuma and Hino (2000) reported that the optimal pH for *S. bovis* PFL was 7.5, much above the pH 5.5 described as optimal for lactate dehydrogenase (LDH) of *S. bovis* strains. However, when the pH of the continuous culture was maintained at 7.5, the fermentation pattern of *S. bovis* HC5 changed considerably. At lower dilution rates, glucose was fermented through a mixed acid pathway.

Because the intracellular concentration of FDP is expected to be reduced at lower fermentation rates, the levels of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate will not be inhibitory to PFL (Garrigues *et al.,* 1997; Asanuma and Hino, 2000). Therefore, acetate, formate and ethanol will be produced in addition to lactate. Homolactic fermentation resumed at lower levels at pH 7.5, but only if the dilution rate was increased to 1.20 h^{-1}.

In this work, we noted that maintaining the culture pH at 7.5 increased glucose utilization when compared to the treatments without pH control. The amount of lactate produced was approximately equal or higher than uncontrolled pH conditions, but at pH 7.5 acetate, formate and ethanol were also produced. Garrigues *et al.,* (1997) demonstrated that the shift from homolactic to mixed-acid fermentation in *L. lactis* was directly correlated to the flux through glycolysis. “Homolactic behavior was seen only during rapid growth in which significant amounts of glucose remained in the medium; mixed-acid fermentation was observed at lower rates of growth and true carbon-limited chemostat steady states” (Garrigues *et al.,* 1997).

S. bovis can assimilate glucose using a phosphotransferase transport system (PTS) (Martin and Russell, 1987) and a low-affinity, facilitated diffusion mechanism is responsible for glucose transport at high substrate concentrations (Russell, 1990). Previous studies indicated that pH can affect the activity of these transport systems (Leonard and Saier, 1983; Andersen *et al.*, 2009), and *S. bovis* JB1 cells had little PTS^{glc} activity when cultivated in batch cultures at pH 5.2. In this latter study, the authors showed that the optimum PTS^{glc} activity occurred at pH 7.2 (Moore and Martin, 1991). Andersen *et al.* (2009) argued that a major cause of the decrease in the glycolytic rate in *L. lactics*, upon lowering the extracellular pH, is the lower pool of phosphoenolpyruvate available to fuel glucose uptake via the phosphoenolpyruvate-dependent transport system.

These results suggest that in continuous cultures at pH 7.5 more glucose was transported into *S. bovis* HC5 cell due to optimum activity of the PTS system. Although the carbon flux through glycolysis is expected to increase favoring homolatic fermentation, PFL activity is at optimal levels, which leads to the production of acetate and formate.

Bovicin HC5 production was higher at lower specific growth rates when the culture pH was left uncontrolled. Many other bacteriocinogenic strains such as *Lactobacillus plantarum* LL441 (Bárcena *et al.,* 1998) and *Eterococcus feacium* (Parent *et al.*, 1997) also showed high levels of bacteriocin production in low continuous culture dilution rates. Lower specific growth rates or suboptimal growth conditions may increase bacteriocin production by lactic acid bacteria (De Vuyst *et al.,* 1996; Aasen *et al.*, 2000; Van den Berghe *et al.*, 2006). It has been suggested that under sub-optimal conditions the consumption rate of available nutrients will be lower and the availability of essential metabolites (including ATP) for bacteriocin biosynthesis will increase

(Aasen *et al.*, 2000). Considering that bacteriocin production constitutes a competitive advantage for the cell (Cleveland *et al.*, 2001), lower specific growth rates may favor bacteriocin production, but studies are needed to investigate the metabolic signal that regulates this process.

When the pH was maintained at 7.5, bovicin HC5 production increased at high dilution rates, but maximum production was at least 10 times lower when compared with uncontrolled pH conditions. At neutral pH, bovicin HC5 is mostly cell-associated (Houlihan and Russell, 2006) and more bacteriocin is expected to be found in the cell extract. Preliminary results suggested that extraction at 100°C was more effective than mild (room) incubation temperatures to extract bovicin from cells. These results indicated that growth conditions could not only influence bacteriocin production but also bacteriocin extraction from cells.

Bacteriocin production by *S. bovis* HC5 at uncontrolled pH condition increased as biomass and lactate production increased and decreased at higher culture pH and lower glucose consumption. However, opposite results were obtained when the pH was maintained at 7.5. These results suggest that bovicin HC5 production is not always growth associated and is affected by extracellular pH.

Our previous results in batch culture showed that the bovicin HC5 production was remarkably increased at pH 5.5. Similar optimal pH values have been described for enterocin 1146, nisin, pediocin and lacticin 481 (Parente and Ricciardi, 1994; Guerra and Pastrana, 2003; Hindré *et al.*, 2004). Bovicin HC5 production in continuous culture was enhanced at acidic conditions and three times more cell-associated bovicin HC5 was recovered from *S. bovis* cells compared to batch systems. Because all assays were performed using acidic NaCl extracts, production rather than activity was primarily affected by the growth conditions.

In this study, we could extract high concentrations of bovicin HC5 from *S. bovis* cells even after a second extraction procedure. Although several strategies have been tested for bovicin HC5 extraction, acidic salt solutions appear to provide preparations with higher yields and less contamination with cell proteins. However, significant amounts of bacteriocin are not being extracted from cells and an increase in yield could be attained simply using multiple extractions or by altering the interaction of the bacteriocin with the cell envelope.

4.6. Conclusions

Continuous culture dilution rates affect bacteriocin production by *S. bovis* under controlled and uncontrolled pH. Fermentation is primarily homolactic under uncontrolled pH and mixed-acid under conditions of controlled pH. Cell-free bovicin HC5 activity is higher in cultures growing slowly at acidic pH or in cultures growing fast at near neutral pH values. Our results showed that pH affect bovicin HC5, but the physiological mechanisms involved in this effect needs to be clarified.

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CHAPTER 5

Isolation of bac$^-$ mutants of *Streptococcus bovis* HC5

5.1. Abstract

Bacteriocins are gene encoded antimicrobial peptides that can be genetically organized in operons located in the chromosome or in mobile elements, such as plasmids and transposons. This work was performed to isolate bac$^-$ mutants and to characterize the location of the structural gene of bovicin HC5. *S. bovis* HC5 cells were transferred 40 times in basal media and bovicin HC5 production showed to be a stable phenotype. No plasmids were detected in *S. bovis* HC5 genome, indicating that the bovicin HC5 biosynthetic genes are chromosomally encoded. The frequency of transformation of *S. bovis* HC5 cells with the pGh9:ISS1 vector was $1.67x10^{-8}$ and the level of chromosomal integration was 0.48%. A total of 30 mutants that showed reduced capacity to produce bovicin HC5 were selected and characterized. The presence of the

vector used in insertional mutagenesis in the genome of some mutants was confirmed by molecular techniques and these mutants can be used to characterize the bovicin HC5 genetic determinants.

Key words: *Streptococcus bovis* HC5, bovicin HC5, insertional mutagenesis, pGh9:ISS1.

5.2. Introduction

Bacteriocins are gene encoded, ribossomally synthesized antimicrobial peptides with several genes involved in their biosynthesis (Chatterjee *et al.*, 2005). These genes can be chromosome encoded or be located in plasmids or transposons (Chatterjee *et al.*,2005; Cotter *et al.*, 2005). Analysis of the genetic determinants of several bacteriocins revealed that genes involved in bacteriocin biosynthesis are organized in one or three operon-like structures (McAuliffe *et al.*, 2001; Chatterjee *et al.*, 2005).

Lantibiotic bacteriocins are characterized to have post-translationally modified aminoacid residues (Chatterjee *et al.*, 2005). Nisin is the most studied lantibiotic and eleven genes are involved in its biosynthesis. This includes the structural gene (*nis*A), proteins involved in posttranslational modification (*nis*BC), transport (*nis*T) and processing (*nis*P) of the peptide, regulation (*nis*RK) of production and immunity (*nis*IFEG) of the producer strain (Kuipers *et al.*, 1998; McAuliffe *et al.*, 2001; Chatterjee *et al.*, 2005).

The antimicrobial peptide bovicin HC5 belongs to the lantibiotic family of bacteriocins and is produced by the ruminal bacterium *Streptococcus bovis* HC5 (Mantovani *et al.*, 2002). *S. bovis* HC5 is a non-fastidious rapidly growing lactic acid

bacterium (Mantovani *et al.*, 2002) that is able to produce bacteriocin over a wide range of culture conditions (Mantovani and Russell, 2003). Bovicin HC5 is a 2440 Da bacteriocin that has a broad spectrum of activity (Mantovani *et al.*, 2002) and potential for application in food safety and veterinary medicine (Carvalho *et al.*, 2007ab; Carvalho *et al.*, 2008; Pinto *et al.*, 2008; Lima *et al.*, 2009).

Unlike nisin, bovicin HC5 is not approved for commercial use. Several studies have been carried out to characterize this bacteriocin, including studies to evaluate its antimicrobial activity (Carvalho *et al.* 2007ab; Carvalho *et al.*, 2008, Pinto *et al*., 2008), production (Carvalho *et al.*, 2009) and citotoxic effect against mammalian cells (Paiva, 2007). However, the genetic characterization of the bacteriocin is needed to study the regulation of bovicin HC5 production and to fully understand its mode of action.

In a previous work, Maguin *et al.* (1996) developed a vector named pGh9:ISS1 that has been used to inactivate several genes of different strains of lactic acidic bacteria. Fontaine *et al.* (2007) utilized this vector to characterize the genes involved in quorum-sensing regulation of bacteriocin production by *Streptococcus thermophilus*. At this work we aimed to isolate bac$^-$ mutants of *S. bovis* HC5 by insertional mutagenesis using this vector. The mutants obtained can be further used to characterize the bovicin HC5 genetic determinants.

5.3. Materials and Methods

5.3.1. Microorganisms and growth conditions

Streptococcus bovis HC5 and *S. bovis* JB1 were cultivated as previously described (Mantovani and Russell, 2003) in basal media containing (per liter): 292 mg

K_2HPO_4, 292 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4.7H_2O$, 64 mg $CaCl_2.2H_2O$, 500 mg cysteine hydrochloride, 1 g Trypticase®, 0.5 g yeast extract, 4 g Na_2CO_3 and 16 g glucose. The medium was prepared anaerobically under an O_2-free carbon dioxide flux and the final pH was adjusted to 6.5 with NaOH (1 mol l^{-1}). Mutantes strains were maintained in M17 medium (Sigma) supplemented with 2 µg of erythromycin per millilitre at 42°C.

The indicator strain *Lactococcus lactis* ATCC 19435 was cultivated aerobically at 37°C in MRS broth (HIMEDIA, Mumbai, India). *Clostridium sticklandii* was grown anaerobically in a basal medium containing salts, vitamins, and minerals (Cotta and Russell, 1982), supplemented with Trypticase® (15 mg ml^{-1}). *Alicyclobacillus acidoterrestris* DSMZ 2498, was grown at 40ºC in *Alicyclobacillus acidoterrestris* medium (AAM), described by Yamazaki *et al.* (2000). *Escherichia coli* strain VE6839 containing the vector pGh9:ISS1, consisting of a temperature-sensitive pG1host replicon, a lactococcal insertion sequence, ISS1, and an erythromycin resistance marker (Maguin *et al.*, 1996), was provided by the INRA National Culture Collection (URLGA, Jouy-en-Josas, France) and was routinely maintained in Luria broth (Sambrock *et al.*, 1989) at 37°C supplemented with 10 µg of kanamycin and 50 µg of erythromycin per millilitre.

5.3.2. Stability of bovicin HC5 production by *Streptococcus bovis* HC5

To verify the stability of bovicin HC5 production, *S. bovis* HC5 were transferred several times in basal media. The incubation temperature was 39°C and the transferences were done after 24 h of incubation. After 1, 5, 10, 15, 20, 30 and 40 transferences, bovicin HC5 was extracted with acidic NaCl solutions from stationary

phase cultures as described by Carvalho *et al.* (2009), with some modifications. *S. bovis* HC5 cells were harvested by centrifugation (9000 *x g*, 4ºC, 15 min) and the cell pellet was re-suspended in NaCl solution (100 mM/pH 2.0) and incubated at 100°C for 20 minutes. Cell suspensions were centrifuged (9000 x g, 4ºC, 15 min) and bacteriocin activity in the cell-free extract was determined. Preparations containing bovicin HC5 were serially diluted (2-fold increments) into NaCl solution (100 mM, pH 2.0) and tested for antimicrobial activity against *L. lactis* ATCC 19435 using the agar well diffusion technique described by Hoover and Harlander (1993). One arbitrary unit (AU) was defined as the reciprocal of the highest dilution that showed a zone of inhibition with at least 5 mm in diameter.

S. bovis HC5 stationary phase cells were diluted (10 times increments) and plated in basal media added with glucose (16 g l^{-1}) and agar (1.5 %). The plates were incubated at 39°C for 24 h. After cell growth, colonies were overlaid with soft MRS agar (0.75%) inoculated with 10^6 CFU ml^{-1} of *L. lactis* cells. The plates were incubated at 4°C for bacteriocin diffusion and then at 37 °C to *L. lactis* growth. Inhibition of *L. lactis* was observed as zone of clearing around *S. bovis* HC5 cells.

To verify if bovicin HC5 was plasmid or chromosome encoded three protocols were used to prepare plasmid from *S. bovis* HC5. The procedures described by Muriana and Klaenhammer (1987) and Rodriguez and Tait (1983) were followed. The protocol to extract large plasmids described by Sambrook *et al.* (1989) was also used, adding lisozyme at 20 mg ml^{-1}.

5.3.3. Electroporation of *S. bovis* HC5 competent cells

Eletrocompetent cells from *S. bo*vis HC5 were obtained as described by Wyckoff and Whitehead (1997). *S. bovis* HC5 was grown aerobically in M17 medium added with glucose and sucrose (SGM17) at 5 g l^{-1} and 171 g l^{-1}, respectively. An aliquota of five ml of the overnight culture was inoculated into 250 ml of SGM17 added with 0.5% glycine and incubated aerobically at 37°C to reach an OD_{600} of 0.2 (approximately 7 h). The culture was placed on ice, and the cells were centrifuged (Sigam 4K15/5000 *x g*/4°C/15 min) and the cell pellet was washed three times in 25 ml of ice-cold 0.5 M sucrose/10% glycerol. The cell pellet was suspended in 0.5 ml of the same solution, and either used immediately for electroporation or stored at -80 °C for future experiments.

Electroporation was carried out in a solution containing sucrose (0.5 M) and glycerol (10%) with a field strength of 2.5 kV/cm, 200 W resistance and 25 µF capacitance. Transformation of *S. bovis* HC5 with plasmid was performed using a gene pulser™ apparatus (Bio-Rad) by adding 100 µl of eletrocompetent *S. bovis* HC5 cells and 500 ng of the pGh9:ISS1 vector in a 0.2-cm electroporation cuvettes (Bio Rad).

The procedures described previously by Maguin *et al.* (1996) were used to obtain *S. bovis* HC5 mutants. Erythromycin-resistant transformants were selected following growth at 28°C. The efficiency of transformation was determined by the ratio of the number of erythromycin-resistant cells divided by the total cell count used for electroporation. Chromosomal integration of pGh9:ISS1 was achieved by 100-fold dilution of an overnight culture of *S. bovis* HC5/pGh9:ISS1 in M17 broth lacking erythromycin (Er) and incubation for 3.5 h at 28°C. The culture was then transferred to 42°C for a further 2.5 h. To determine the transposition frequencies, samples were

diluted and plated at 42°C on M17 agar with or without erythromycin. Transposition frequencies correspond to the number of Er-resistant cells (obtained on Er-containing M17 medium at 42°C) divided by the total cell number (enumerated on M17 medium at 42°C). Cultures were stored in M17 broth added with 15% glycerol at -20°C.

5.3.4. Screening of non-bacteriocinogenic mutants (bac$^-$)

The screening for a bac$^-$ phenotype was carried out using *C. sticklandii* SR and *A. acidoterrestris* DSMZ 2498 as indicators of bacteriocin production. To verify the activity of bovicin HC5 against *C. sticklandii*, erythromycin-resistant *S. bovis* HC5 cells were washed and inoculated (approximately 10^9 CFU ml^{-1}) in minimal medium lacking glucose. Trypticase® was added at 15 mg ml^{-1} and *C. sticklandii* SR cells at 2% inoculum (v/v) (Xavier *et al.*, 2008) and ammonia production by the indicator organism was determined using a colorimetric assay (Chaney and Marbach, 1962). The wild type strain *S. bovis* HC5, *S. bovis* JB1, a non-bacteriocinogenic strain and *C. sticklandii* were used as controls.

Mutants were screened for antimicrobial activity against *A. acidoterrestris* based on a agar overlay procedure where mutants were spotted onto GM17 agar plates and incubated at 42°C for 24 h. Colonies were then overlayed with soft AAM (0.7% agar) containing approximately 10^5 CFU ml^{-1} of an actively growing culture of the indicator organism and after 12 h of incubation at 40°C the colonies were screened for the presence of zones of inhibition. The isolates that did not inhibit ammonia production by *C. sticklandii* SR (bac$^-$) or/and did not show an inhibition zone against *A. acidoterrestris* were selected for further analysis.

The bac$^-$ mutants selected were grown on basal media and submitted to gram staining and fermentation acids were analyzed in cell-free supernatant by high performance liquid chromatography (HPLC, HPX-87H column, Bio-Rad, Hercules, CA). The sample size was 20 µl, the eluant was 0.005 M H_2SO_4, the flow rate was 0.7 ml min^{-1} and the column temperature was 60ºC. The bac$^-$ mutants were inoculated into M17 broth and the growth was monitored by determining the optical densities at 600 nm with a microplate reader (TP reader, Thermo Plate, Waltham, MA). The specific growth rate was also determined for each isolate.

5.3.5. DNA techniques

S. bovis HC5 genomic DNA from wild type and mutant strains and the vector pGh9:ISS1 from *E. coli* VE6839 were prepared using the Wizard Genomic DNA purification Kit A1125 (Promega, Madison, WI, USA) and the Plasmid Maxi Kit (Qiagen, Hilden, Germany), respectively, following manufacturer recommendations. To verify the presence of the pGh9:ISS1 vector in the genome of bac$^-$ mutants, PCR amplification of the ISS1 element was performed using the primers ISS1for (5'-GGAGAGAATGGGTTCTGTTGC-3') and ISS1rev (5'-GCTCTAGAGCATTCTCTGGTTC-3') designed by Ward *et al.* (2001). PCR was performed under the following conditions: 94°C for 1 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, and 72°C for 5 min. Amplification of DNA was performed using an programmable thermal controller model PTC-100^{TM} (MJ Research, Watertown, Mass., USA).

Southern blot, DNA digestion and ligase reactions followed standard procedures (Sambrook *et al.*, 1989) and the manufacture's instruction. To Southern blot analyzes,

genomic DNA (3 μg) isolated from wild type strain and mutants were digested using *Hind*III (which does not cut inside the ISS1 element used as a probe), transferred to a nylon membrane (RPN303B, Amersham Biosciences) and hybridized with the ISS1 at a high stringency condition (65°C). The ISS1 probe was labeled with alkaline phosphatase using the direct labeling and detection system (GE Healthcare/Amersham Gene Images/RPN36). The ISS1 sequence was obtained by PCR amplification using the pGh9:ISS1 vector as a template as described above. All enzymes used in this study were obtained from Promega (Madison, WI, USA).

5.4. Results

5.4.1. Stability of bovicin HC5 production by *Streptococcus bovis* HC5

When *S. bovis* HC5 cells were transferred to basal media (16 g l^{-1} glucose), cell-associate bovicin HC5 activity increased as the number of transfers increased (Figure 5.1a). The bovicin HC5 activity doubled after each five transfers up to the 15th transfer (Figure 1a). In the 15th transfer, bovicin HC5 activity was approximately 80 percent higher compared to the first transfer (Figure 5.1a). After 20 transfers, the activity of bovicin HC5 stabilized at 10.240 AU ml^{-1} (Figure 5.1a). When *S. bovis* HC5 colonies were overlaid with soft agar inoculated with *L. lactis* cells, all colonies showed inhibitory activity against to this strain (Figure 5.1b). Plasmids were not found in the genome of *S. bovis* HC5, even if three different protocols were used to extract plasmids from *S. bovis* cells (Figure 5.1c).

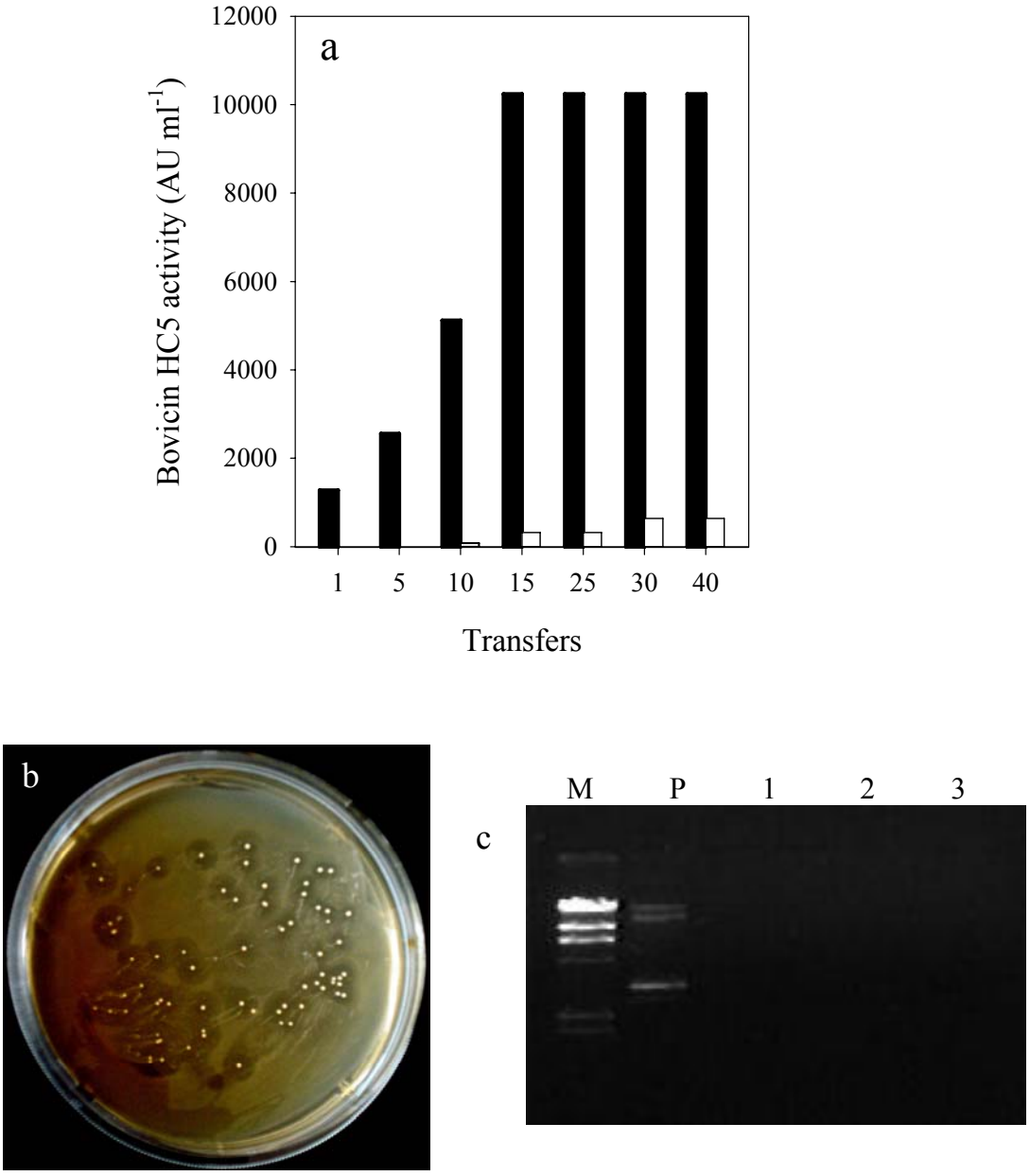

Figure 5.1. Stability of bovicin HC5 production by *S. bovis* HC5. a) Batch cultures of *S. bovis* HC5 cells were successively transferred in basal media (16 g l^{-1} glucose) and cell-associated (closed bars) and cell-free (open bars) bovicin HC5 activity were determined at time intervals using *L. lactic* ATCC 19435 as the indicator strain. b) Cells of *S. bovis* HC5 were diluted and plated in basal media. The colonies were overlaid with *L. lactis* cells and zone of inhibition were observed around colonies. c) Three different protocols were tested to extract plasmids from *S. bovis* HC5 cells. Lanes 1, 2 and 3 corresponding to methods described by Muriana and Klaenhammer (1987), Rodriguez and Tait (1983) and Sambrook *et al.* (1989) respectively. Lane M contains the λ *Hind*III marker and lane P the pGh9:ISS1 vector.

5.4.2. Isolation of bac$^-$ mutants

The frequency of transformation of *S. bovis* HC5 cells with the temperature-sensitive plasmid pGh9:ISS1 was 1.67×10^{-8}. Chromosomal integration of pGh9:ISS1 was achieved by subsequent growth of erythromycin-resistant *S. bovis* HC5 cells at the non-permissive temperature, and the level of integration was 0.48%. A total of 244 colonies with the pGh9:ISS1 vector integrated in the genome were isolated. Among these, 30 isolates had little inhibitory activity against *C. sticklandii* SR when compared to the wild type *S. bovis* HC5 cells (Table 5.1). Although the mutants varied in their ability to inhibit ammonia production by *C. sticklandii* SR, deamination was always higher than the positive control (*S. bovis* HC5) (Table 5.1). Some mutants that did not show inhibitory activity against *A. acidoterrestris* still inhibited ammonia production by *C. sticklandii* (Table 5.1).

Table 5.1. Phenotypic characterization of bac$^-$ mutants of *S. bovis* HC5

Mutant Strain	**Specific growth rate (h^{-1})**	**Fermentation product**	**Inhibition of *A. acidoterrestris* (presence of inhibition zone)**	**Inhibition of ammonia production by *C. sticklandii* (%)**
HC6	0.34	lactate	+	11
HC7	0.38	lactate	+	0
HC8	0.35	lactate	+	7
HC9	0.36	lactate	+	0
HC10	0.27	lactate	+	0
HC11	0.36	lactate	-	6
HC12	0.48	lactate	-	8
HC13	0.48	lactate	-	0
HC14	0.47	lactate	-	0
HC15	0.52	lactate	+	15
HC16	0.35	lactate	+	5
HC17	0.33	lactate	+	0.5
HC18	0.48	lactate	+	34
HC19	0.47	lactate	+	5
HC20	0.48	lactate	-	2
HC21	0.49	lactate	-	0
HC22	0.47	lactate	-	0
HC23	0.50	lactate	-	22
HC24	0.50	lactate	-	15
HC25	0.53	lactate	-	14
HC26	0.57	lactate	-	36
HC27	0.48	lactate	-	39
HC28	0.50	lactate	-	20
HC29	0.50	lactate	+	35
HC30	0.53	lactate	+	32
HC31	0.51	lactate	-	15
HC32	0.55	lactate	-	41
HC33	0.60	lactate	-	10
HC34	0.55	lactate	+	39
HC35	0.51	lactate	-	15
		Controls		
HC5	0.95	lactate	+	72
JB1	0.90	lactate	-	17

5.4.3 Characterization of mutants

The specific growth rate of mutants was approximately 50% lower than the specific growth rate of the wild type strain (Table 5.1). The integrated mutants were grown in basal media added with 4 g l^{-1} of glucose and lactate was the only fermentation product, as observed for the wild-type strain (Table 5.1). Mutants that showed greater specific growth rates were selected for PCR amplification of the ISS1 fragment present in the vector used for insertional mutagenesis. A total of 22 mutants were analyzed, and six mutants presented negative PCR results (Figure 5.2). However, amplified products of the expected size (1.4 kb) were obtained for 16 mutants, confirming the presence of the ISS1 element in the genome of the bac^- strains (Figure 5.2).

These mutants were also analyzed with dot blot using ISS1 as a probe. Hybridization with the ISS1 sequence was observed for eight mutants tested (Figure 5.3). Six of the dot blot-positive mutants were submitted to Southern blot analysis of the *Hind*III-digested genomic DNA. The results showed that the ISS1 sequence was present in four DNA from mutants and absent from two previously selected mutants and in the parental strain *S. bovis* HC5 (Figure 5.4). In 3 of the mutants, single copies of the ISS1 element were detected in fragments of approximately 4 and 1 kb (Figure 5.4). The remaining mutant showed two copies of the ISS1 element in the genome in fragments of approximately 4 and 8 kb (Figure 5.4).

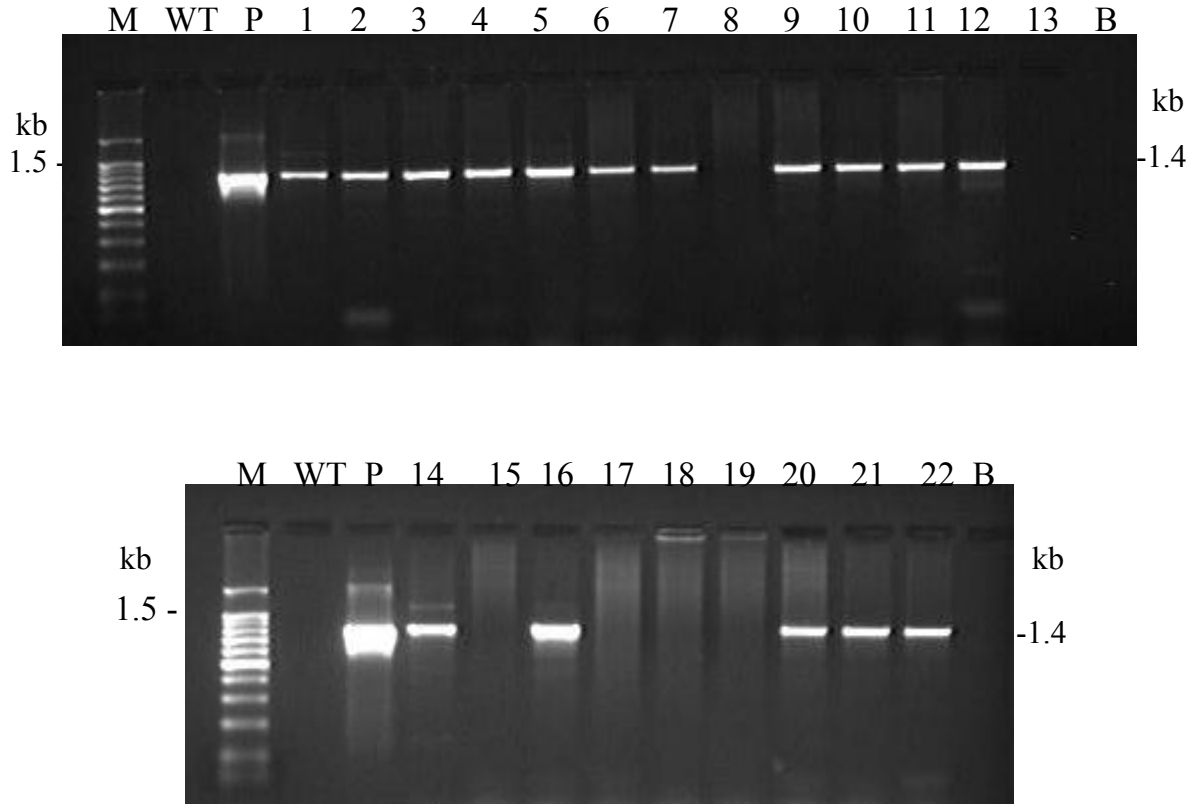

Figure 5.2. Agarose gel electrophoresis (1.5%) of the ISS1 fragment (1.4 kb) PCR-amplified by using primers ISS1for and ISS1rev. Numbers 1 to 22 indicate *S. bovis* HC5 mutant DNA. (M) molecular size marker 100 pb ladder, (WT) wild type, (P) pGh9:ISS1 vector (B) negative control: reaction mix without DNA.

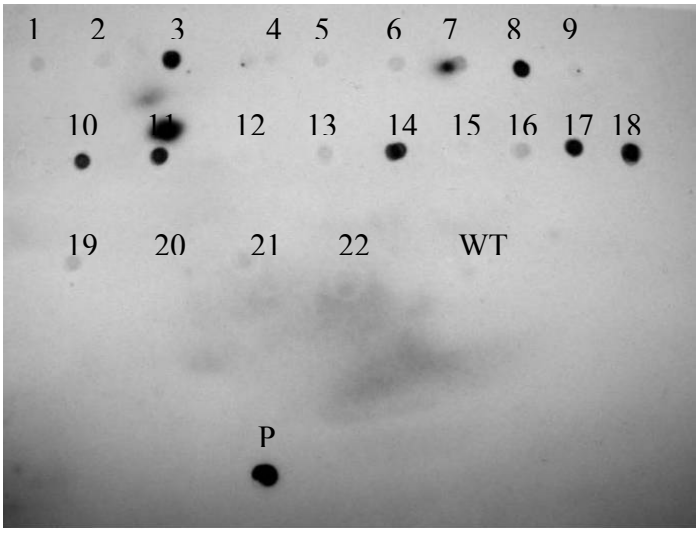

Figure 5.3. Autoradiography of dot blot analyzis from mutants. DNA preparations of mutants (number 1 to 22, 250 ng), wild type strain (WT, 250 ng) and pGh9:ISS1 vector (P, 20 ng) were denatured by NaOH (4 M) and added to nylon membrane. The membrane was treated at high stringency condition (65 °C) with the ISS1 probe directly labeled with alkaline phosphatase and exposed to an autoradiographic film.

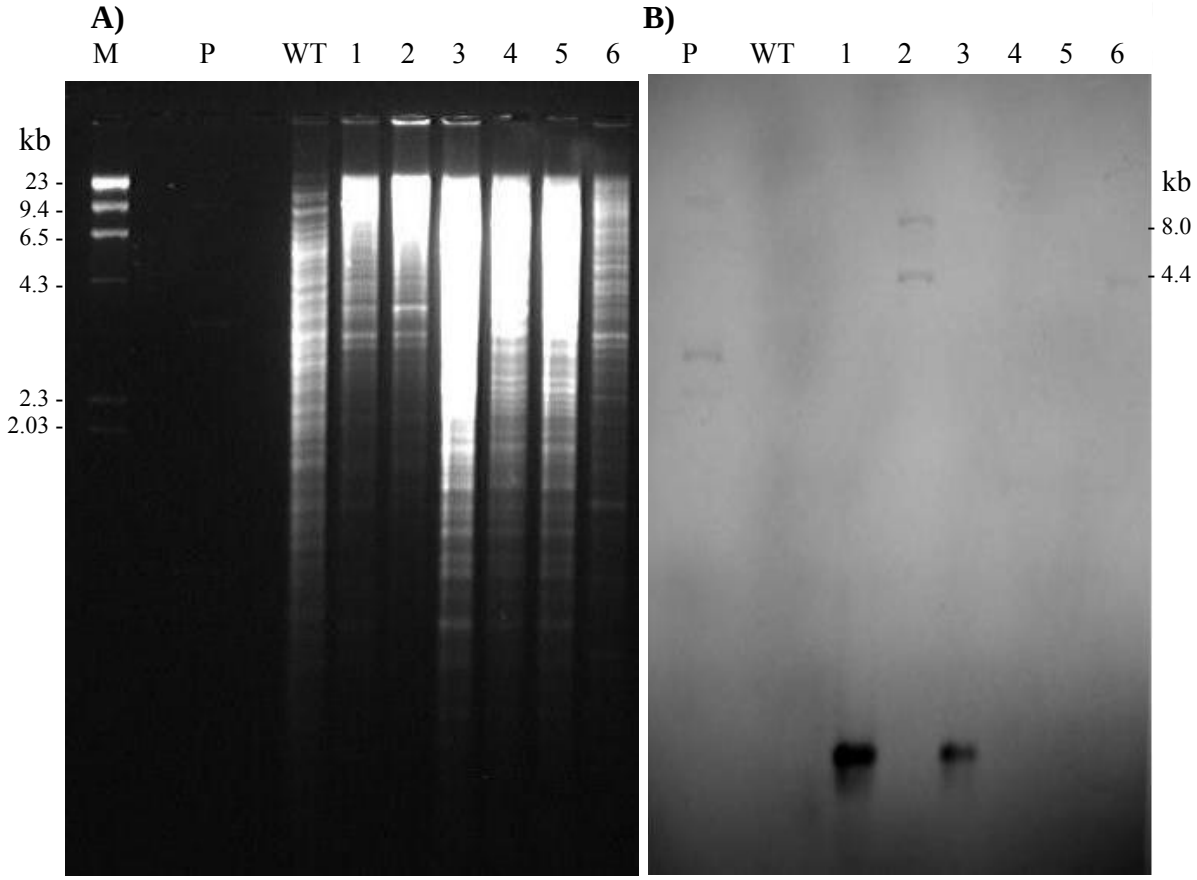

Figure 5.4. Southern-blot analysis of total DNA from *S. bovis* HC5 wild type strain and mutants. A) Agarose gel electrophoresis (0.8%) of genomic DNA from the wild type strain (WT) and mutants (lanes 1 to 6) digested using *Hind*III. B) Autoradiographic film of hybridization at 65 °C of total DNA from mutants (lanes 1 to 6) and wild type strain (WT) using the ISS1 fragment as a probe. The vector pGh9:ISS1 was used as a positive control (P). Lane M contains the λ *Hind*III marker. The sizes of bands are showed in kb.

5.5. Discussion

Bovicin HC5 is a lantibiotic bacteriocin with potential for commercial use (Pinto *et al.*, 2008; Carvalho *et al.* 2008; Lima *et al.*, 2009). However, its genetic characterization is lacking and little information is known about the organization of the genes involved in biosynthesis. Studies with lantibiotic gene clusters have shown that the genes involved in lantibiotic production may be chromosomal or plasmid encoded and includes the structural gene and several genes involved in modification, transport, processing, regulation and immunity (Siezen *et al.*, 1996; McAuliffe *et al.*, 2001; Chatterjee *et al.*, 2005).

Results indicated that bacteriocin production by *S. bovis* HC5 is a stable phenotype, as no plasmids were detected in the genome of *S. bovis* HC5. These results suggest that the bovicin HC5 biosynthetic genes are chromosome encoded. The bovicin HJ50 gene cluster, a bacteriocin produced by *Streptococcus bovis* HJ50, was also found to be located in the chromosome of the producer strain (Xiao *et al.*, 2004; Liu *et al.*, 2009).

When *S. bovis* HC5 cells were transferred several times in basal media, the activity of bovicin HC5 increased as the number of transfers increased. Greater nisin yields were obtained when a plasmid containing genes for the immunity protein (NisI) was introduced into *Lactococcus lactis* cells (Kim *et al.*, 1998). Considering that the *S. bovis* HC5 transfers were done after the cells had reached stationary phase of growth, cells with higher immunity proteins could be naturally selected, thus explaining the increase in bovicin HC5 recovered from the cells.

Transposition has been a valuable genetic tool to study chromosomal genes, their functions, and their regulators in many bacteria. Maguin *et al.* (1996) developed a

highly efficient transposition mutagenesis system based on the delivery of ISS1 by the thermosensitive plasmid pG1host for lactococci, and this system have been successfully applied to many lactic acid bacteria (Ward *et al.*, 2001; Mora *et al.*, 2004; Fontaine *et al.*, 2007). In this study, a transposition mutagenesis system was used to obtain bac$^-$ mutants of *S. bovis* HC5. The vector suffered efficient insertion, and among the integrated mutants obtained, approximately 12% showed a non-bacteriocinogenic phenotype.

The bac$^-$ mutants had the same profile of glucose fermentation as the wild type strain. However, the specific growth rate of bac$^-$ mutants was lower than the wild type. This result could be explained by the insertion of the pGh9:ISS1 vector in genes that are either required for bacteriocin production and for *S. bovis* HC5 growth or by the integration at multiple sites. Integration of pGh9 vector in the genome has been shown to occur predominately as mono-copy, representing approximately 80% of the transposition events (Maguin *et al.*, 1996; Ward *et al.*, 2001). Southern analyzes confirmed simple transposition of the pGh9:ISS1 vector in the *S. bovis* HC5 genome, resulting in the mutant strains.

Another explanation to the reduced specific growth rate of *S. bovis* mutants is that the insertion of a 6 kb fragment in the chromosome affected the time of DNA replication and, consequently, the rate that mutants grew. The presence of the pGh9:ISS1 vector in *S. bovis* chromosome affected only the specific growth rate. At the end of the logarithmic growth phase, the optical density of the mutant and wild type strains leveled off at similar values (approximately 3.0) (not shown results).

The mutants showed different degrees of antimicrobial activity against *C. sticklandii* and *A. acidoterrestris*. These results also support the idea that the insertion of the pGh9:ISS1 vector in *S. bovis* HC5 chromosome might have inactivated other genes

that are directly or indirectly involved in bovicin HC5 biosynthesis. Since several genes are involved in lantibiotic biosyntheses (McAuliffe *et al.*, 2001; Chatterjee *et al.*, 2005), the inactivation of any of these genes may result in different levels of bacteriocin expression. For example, at least two types of immunity genes have been described: an immunity protein that protect the cell against bacteriocin activity and a transport system that pump the peptide out of the cell (Cleveland *et al.*, 2001; Chatterjee *et al.*, 2005). If one of these genes is inactivated, the immunity level of the producer strain will decrease as well as the production of the bacteriocin. However, the antimicrobial activity will not be completely abolished since the other immunity system remains functioning. As observed for *Lactococcus lactis*, the increase in the expression of the immunity protein NisI increased nisin production (Kim *et al.*, 1998) and an opposite situation can also occur.

We have not yet been able to explain the fact that erythromycin resistant mutants submitted to PCR amplification of the ISS1 element were positive for the presence of the ISS1 element but negative for hybridization when assayed by dot and Southern analysis. For further characterization of the mutants, we only considered the strains showing positive results during Southern analysis in order to pursue the sequencing of the structural gene. One mutant (Figure 5.4, lane 2) presented two bands, one of 4 kb and another with 8 kb. The presence of these two bands indicate the presence of the pGh9 element flanked on either side by ISS1, due to replicative transposition, a characteristic of the ISS1 element (Maguin *et al.*, 1996). From the other three mutants that rendered positive results, only one band was observed (Figure 5.4). In lane six, a band of approximately 4 kb was observed. This fragment was amplified by inverse PCR and sequenced. Because sequencing showed that the pGh9 vector was present (data not shown) in this DNA fragment, we concluded that this line might have had a second but

very weak band that could not be readily detected. Lanes one and three in figure 5.4 showed bands with less than 2 kb corresponding to the ISS1 element. These results suggest the occurrence of recombination events in *S. bovis* HC5 genome. Considering that to extract DNA from mutants the growth temperature was restrictive to the pGh9:ISS1 vector, these sequences might be integrated in the *S. bovis* bac$^-$ mutants.

5.6. Conclusion

The results obtained suggested that the bovicin HC5 biossynthetic genes are chromosomally encoded. The pGh9:ISS1 was efficient to obtain *S. bovis* HC5 cells that do not produce bacteriocin and could be used as a molecular tool to study several other genes in this microorganism. The mutants obtained in this study can be used to characterize the bovicin HC5 genetic determinants.

5.7. References

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