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# LIKE IT ACID AND POOR: A STUDY OF ABIOTIC FACTORS INFLUENCING *Streptococcus bovis* HC5 GROWTH AND BACTERIOCIN PRODUCTION

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**ABSTRACT**

This study aimed to investigate the effect of pH, temperature, growth atmosphere and nutrient availability on bovicin HC5 production by *Streptococcus bovis* HC5. *S. bovis* HC5 grew well in complex and basal media under aerobic and anaerobic conditions, but greater bacteriocin yields were recovered from anaerobic cultures. Lactate production and glucose consumption increased if *S. bovis* HC5 cells were cultivated at pH 7.0 and at 45 ºC, but higher bovicin HC5 activity was recovered from cells grown in acidic conditions and at lower temperatures (39 ºC). Cultures maintained under continuous $CO_2$ flow showed faster growth rates in basal media, but bacteriocin production was always higher if *S. bovis* was cultivated in anaerobic sealed tubes. These results suggest that acidic pH and anoxic conditions favor bovicin HC5 production by *S. bovis* HC5. *S. bovis* HC5 is a unique lactic acid bacterium in its ability to grow and produce high amounts of a potentially useful bacteriocin in simple media. Considering the constrains for bacteriocin production at commercial scale, it appears that bovicin HC5 production could be achieved at lower costs compared to other bacteriocins from lactic acid bacteria.

**Keywords:** Bovicin HC5, pH, environmental conditions, lactic acid bacteria

## 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 of these antimicrobial peptides are effective in controlling spoilage microorganisms in foods or inhibiting human and animal pathogens **(Bowe *et al*. 2006; Coelho *et al*. 2007; Carvalho *et al.* 2008; Hartmann *et al.* 2011; Crowley *et al*., 2013; Sharma *et al*., 2013; Barbosa *et al*., 2013)**. Since these characteristics are useful for the pharmaceutical and food industries, there is a great interest in expanding the characterization and production of antimicrobial peptides with potential for commercial applications.

The increase in antibiotic-resistant pathogens has also stimulated the study of bacteriocins as an alternative to classical antibiotic to treat infectious diseases and reduce the risk of selecting antibiotic-resistant strains **(Brumfitt *et al*., 2002; Rea *et al*., 2007; Piper *et al*., 2009).** Bovicin HC5, a lantibiotic produced by *Streptococcus bovis* HC5 has wide spectrum of activity, stability to heat and acidic pH and uses lipid II as its target in the cell membrane **(Mantovani *et al*., 2002; Houlihan *et al*., 2004; Paiva *et al*., 2011)**. Previous studies indicated that bovicin-resistance is not a phenotype easily selected among sensitive bacteria **(Mantovani *et al*., 2001; Mantovani and Russell, 2003a; Carvalho *et al*., 2007ab).** Because of its characteristics, bovicin HC5 has been suggested as a promising peptide to be used for commercial applications **(Carvalho *et al*., 2009; Lima *et al*., 2010).**

In order to be considered for practical applications, large scale production of relatively pure bacteriocins must be obtained and the factors influencing bacteriocin production must be determined. In a previous work, *S. bovis* HC5 was able to produce bovicin HC5 under a wide range of culture conditions **(Mantovani and Russell 2003b),** and more recent results indicated that carbon and nitrogen sources affect the amount of cell-free and cell-associated bovicin HC5 **(Carvalho *et al*., 2009)**.

Considering that *S. bovis* HC5 is a facultative anaerobic ruminal bacteria **(Mantovani *et al*., 2001)**, and the fact that environmental factors and media composition affect bacteriocin production by lactic acid bacteria **(Van Den Berghe *et al*., 2006; Carvalho *et al*., 2009; Aguilar-Uscanga *et al*., 2013)**, this work aimed to investigate the effect of pH, temperature, aeration and different media on bovicin HC5 production by *S. bovis* HC5.

## MATERIAL AND METHODS

### Microorganisms and culture media

The bacteriocin producer strain *Streptococcus bovis* HC5 was isolated from the rumen of cattle (Mantovani et al. 2001) and cultivated as previously described **(Mantovani and Russell, 2003b)** in basal media containing (per litre): 292 mg $K_2HPO_4$, 292 mg $KH_2PO4$, 480 mg $(NH4)_2SO_4$, 480 mg NaCl, 100 mg, $MgSO_4.7H_2O$, 64 mg $CaCl_2.2H_2O$, 500 mg cysteine hydrochloride, 1.0 g Trypticase, 0.5 g yeast extract, 16 g glucose and 4.0 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 $L^{-1}$).

The indicator organism, *Alicyclobacillus acidoterrestris* DSMZ 2498, was grown at 40 ºC in *Alicyclobacillus acidoterrestris* medium (AAM), described by **Yamazaki *et al.* (2000)**. AAM was composed of (per liter): yeast extract, 1.0 g, $(NH_4)_2SO_4$, 0.2 g, $MgSO_4•7H_2O$, 0.5 g, $CaCl_2•2H_2O$, 0.25 g, $KH_2PO_4$, 1.0 g, glucose, 1.0 g, and distilled water (1000 mL, pH 4.0). Solid medium (AAM agar) was prepared mixing 2-fold concentrated AAM broth (500 mL) with a 4% (w/v) agar stock solution (500 mL) prepared with distilled water. Each solution was heat-sterilized separately (121ºC/15 min) and mixed while the medium was still hot.

### Bovicin HC5 bioassay

Free bovicin HC5 and the cell-associated bacteriocin were determined in cell-free supernatants and in acidic extracts obtained from *S. bovis* HC5 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 by agar well

diffusion **(Hoover and Harlander, 1993)**. Bovicin HC5 activity was estimated from zones of clearing around each well and represented as the reciprocal of the highest dilution that still produced a zone of inhibition with at least 10 mm in diameter. The yield of bovicin HC5 production was calculated by the relation between bacteriocin activity (AU $mL^{-1}$) and biomass produced (mg cell dry mass). Cell dry mass (biomass) from cultures was determined as described by **Carvalho *et al*. (2009)**, where the relationship between optical density (600 nm) and cell dry mass for *S. bovis* HC5 was 360 mg cell dry mass $liter^{-1}$ turbidity $unit^{-1}$. To calculate the biomass from cultures, the OD values were multiplied by 0.360 to obtain results per mL. The specific activity of bovicin HC5 was determined by dividing the activity (AU $mL^{-1}$) by biomass. The activity of bovicin HC5 was expressed as arbitrary units (AU) per mg of cell dry mass (AU $mL^{-1}$ $mg^{-1}$ of cell dry mass).

**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 , 2003b)**. 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 39 ºC and the growth was monitored spectrophotometrically (OD600nm - Spectronic 20D+, Thermo 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 (μ) was calculated from the rate of increase in cell biomass (X) based on the equation $dX/dt = \mu X$, where μ is an absolute rate constant with the units of $h^{-1}$ and t is the growth time of in exponential phase. The specific growth rate ($h^{-1}$) was estimated from the differences between the natural logarithm of optical density and time. The final pH of the cultures were also determined.
*S. bovis* HC5 was also grown in basal media under three different conditions: 1) continuous $CO_2$ flux (CCF), 2) sealed anaerobic tubes (SAT) and 3) aerobic conditions. The changes in optical density and bacteriocin production were monitored during *S. bovis* HC5 growth as described above.

**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 OD600nm in a Spectronic 20D+. In another experiment, the culture pH was maintained at constant values using a pH controller (Model 5656-00, Cole Parmer, Illinois, USA) to maintain the hydrogen ion concentration in a range that *S. bovis* HC5 was able to grow (pH 5.5 to 7.0).
*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 for 16 and 24 hours, samples of 50 mL were withdrawn and OD and bovicin HC5 activity were determined in acidic NaCl extracts and in cell-free supernatants, as described above. Glucose and fermentation acids in cell-free supernatants were analyzed by high performance liquid chromatography (HPLC, Aminex Bio-Rad HPX-87H organic acid column, 300 x 7.8 mm I.D., 9 μm particle size). 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 by 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 of dry cell mass, were determined for all pH and temperature conditions described above.

**Statistics**

All experiments were carried out at least in duplicate and repeated twice. The results obtained were subjected to analysis of variance (ANOVA), and the means were compared by the Tukey test, at 5% of probability, using the SAS statistical software (**Statistical Analysis Systems**, **2004**). When error bars are given in the figures, they refer to the standard deviation of the mean.

**RESULTS**

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

When *S. bovis* HC5 was grown in different media, bovicin HC5 was detected in the cell-free supernatant and in the acidic NaCl extracts obtained from all conditions tested (Tab 1). The specific growth rate and biomass production were always greater if growth conditions were anaerobic. Except for basal media, faster growth and greater biomass were associated with decreased bovicin HC5 production (Tab 1).

**Table 1** Effect of media composition on growth parameters of S*treptococcus bovis* HC5 and specific activity of cell-free (supernantant) and cell associated (extract) bovicin HC5.

| Parameter | Growth condition | Media* | | | |
|---|---|---|---|---|---|
| | | **MRS** | **M17** | **BHI** | **Basal Media** |
| Final pH** | Aerobiosis | 4.53ab | 4.33bA | 4.53ab | 4.60aA |
| | Anaerobiosis | 4.19aB | 4.45bA | 5.26cB | 4.20aB |
| μ ($h^{-1}$) | Aerobiosis | 0.55aA | 0.41bA | 0.59aA | 0.74cA |
| | Anaerobiosis | 0.73aB | 0.75aB | 0.99bB | 1.15cB |
| Cell dry mass (mg $mL^{-1}$) | Aerobiosis | 0.72aA | 0.73aA | 0.50bA | 0.74aA |
| | Anaerobiosis | 1.44aB | 1.35aB | 1.20bB | 1.15bB |
| Cell-free Bovicin HC5 specific activity (AU $mL^{-1}$ $mg^{-1}$ dry cell mass) | Aerobiosis | 888aA | 888aA | 160bA | 432cA |
| | Anaerobiosis | 83aB | 178bB | 133cB | 914dB |
| Cell-associated Bovicin HC5 specific activity (AU $mL^{-1}$ $mg^{-1}$ dry cell mass) | Aerobiosis | 444aA | 333bA | 80cA | 1,730dA |
| | Anaerobiosis | 28aB | 80bB | 67cB | 5,485dB |

**Legend:** * Means for the same parameter followed by at least one same letter, uppercase in column and lowercase in line, did not differ significantly by Tukey test at 5% probability, **Initial pH was 6.5

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 (Tab 1). However, the reverse was observed for the basal media. In this latter treatment, higher cell densities and specific growth rates were related with greater bovicin HC5 production in the cell extract (Tab 1). Because complex media seemed to reduce bacteriocin yield, basal media was chosen to be used in subsequent experiments.

**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 growth rate and final optical density increased at higher pH values (Tab 2). Cell-associated bovicin HC5 specific activity was highest (4923 AU $mL^{-1}$ $mg^{-1}$ dry cell mass) 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 (Tab 2).

**Table 2** Effect of medium pH on growth parameters and bovicin HC5 production by *Streptococcus bovis* HC5

| Initial pH | Final pH* | Final OD* | μ* ($h^{-1}$) | Bovicin HC5 specific activity (AU $mL^{-1}$ $mg^{-1}$ dry cell mass)* | |
|---|---|---|---|---|---|
| | | | | Supernatant | Extract |
| 4.50 | 4.50ab | 0.136e | NG | 0e | 0e |
| 5.00 | 5.00a | 0.13e | NG | 0e | 0e |
| 5.50 | 4.00bc | 0.54d | 0.45d | 400.00a | 1,600.00b |
| 6.00 | 3.80c | 1.25c | 0.82c | 355.00b | 1,422.00c |
| 6.50 | 4.18bc | 2.90b | 0.95b | 307.00c | 4,923.00a |
| 7.00 | 3.00d | 3.79a | 1.15a | 235.00d | 470.00d |

**Legend:** NG = No growth, * Means with the same letter in the column are not significantly different by Tukey test at 5% probability.

When *S. bovis* HC5 was grown in basal medium with pH control, bovicin HC5 specific activity increased with the reduction in the media pH (Tab 3). Higher bacteriocin activity was observed if the pH was maintained at 5.5, independently of the incubation time (Tab 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 (Tab 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 (Tab 3). Bovicin HC5 activity was not detected in the culture supernatant when the pH was maintained at 6.5 and 7.0 (Tab 3).

**Table 3** Effect of controlled pH on bovicin HC5 production by *Streptococcus bovis* HC5. Control treatment without pH control is also shown.

| Parameter | Time (h) | pH* | | | | |
|---|---|---|---|---|---|---|
| | | 5.5 | 6.0 | 6.5 | 7. 0 | Control |
| Biomass (mg $mL^{-1}$) | 16 | 1.17abA | 1.20abA | 1.29aA | 1.02bcA | 0.90cA |
| | 24 | 1.37aA | 1.37abA | 1.39bA | 0.96acA | 1.03cA |
| Supernatant Bovicin HC5 specific activity (AU $mL^{-1}$ $mg^{-1}$ dry cell mass) | 16 | 3,096.00aA | 254.00bA | 0cA | 0cA | 1,536.00dA |
| | 24 | 66,064.00aB | 3,047.00bB | 0cA | 0cA | 604.00dB |
| Cell Extract Bovicin HC5 specific activity (AU $mL^{-1}$ $mg^{-1}$ dry cell mass) | 16 | 23,032.00aA | 14,380.00aA | 14,576.00abA | 1,184.00bcA | 4,960.00cA |
| | 24 | 518,516.00aB | 14,380.00bA | 11,288.00bA | 0cA | 4,490.00bcA |

**Legend:** * Means for the same parameter followed by at least one same letter, uppercase in column and lowercase in line, did not differ significantly by Tukey test at 5% probability.

Control treatment showed similar levels of bovicin HC5 activity in the acidic extract after 16 and 24 hours of incubation, which was also observed for pH 6.0 and 6.5 (Tab 3). However, in pH 5.5 the bacteriocin activity increased more than 20-fold after 24 hours of incubation. Bovicin HC5 was not detected by *S. bovis* HC5 cultures maintained for 24 h at pH 7.0. 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 $mL^{-1}$ (Tab 3). At pH 7.0 and in the controls, lower values of microbial biomass were obtained (Tab 3). When the culture supernatant was analyzed by HPLC, lactate was the only metabolic product of glucose fermentation. Lactate concentration and residual glucose increased at higher pH values (Fig 1).

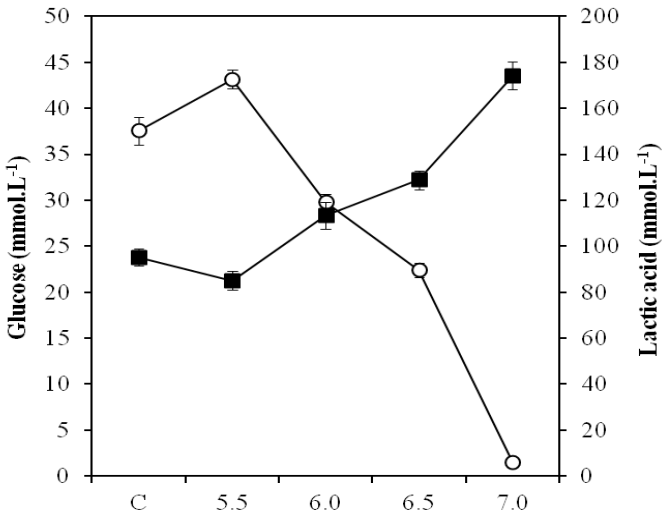


**Figure 1** The consumption of glucose (open circles) and production of lactic acid (closed circles) by *S. bovis* HC5 grown at different pH values. *S. bovis* was inoculated into basal media added with glucose at 16 g $L^{-1}$ and the media pH was maintained at 5.5, 6.0, 6.5 and 7.0. After 24 hours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

If *S. bovis* HC5 was cultivated in basal media (pH 6.5) at different temperatures, growth occurred at temperatures ranging from 30ºC to 45ºC (Fig 2). 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 (Fig 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 in activity of approximately 20% was observed at 45ºC (Fig 2b).

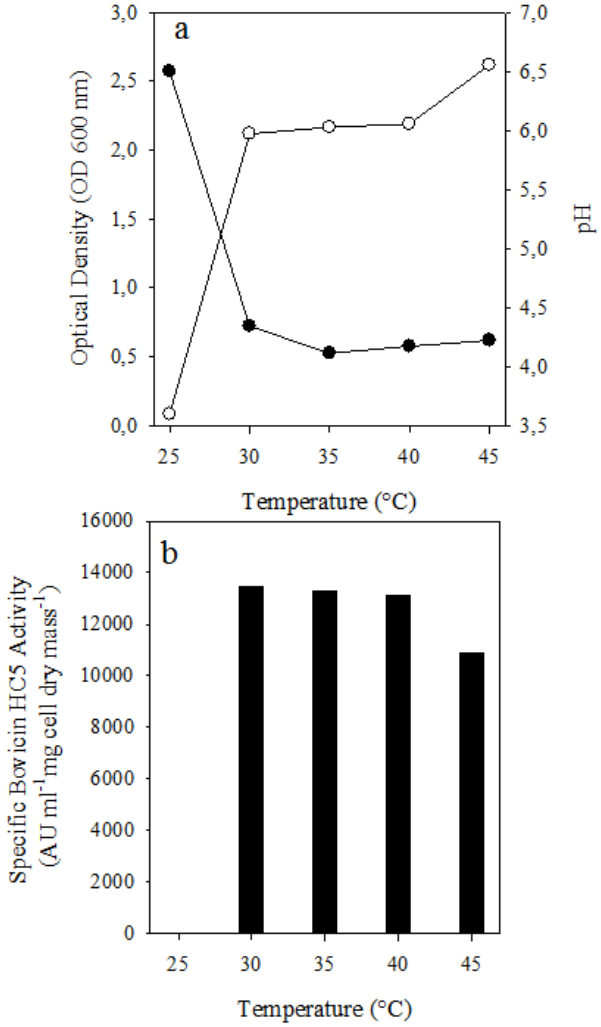


**Figure 2** Effect of incubation temperature on *S. bovis* HC5 growth and bovicin HC5 production. *S. bovis* HC5 was cultivated anaerobically in basal media (pH 6.5) incubated for 24 h at temperatures ranging from 25ºC to 45ºC. (a) The final pH (closed circles) and the OD600 nm (open circles) were measured. The specific activity of cell-associated bovicin HC5 against *A. acidoterrestris* DSMZ 2498 is indicated in (b).

**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 lag phase duration of the bacteriocin producer strain was at least 2 hours (Fig 3). However, the specific growth rate was greater in CCF and maximal biomass production was observed in SAT (Fig 3).

Bovicin HC5 was monitored during *S. bovis* HC5 growth and inhibitory activity was detected for all conditions tested (Fig 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 (Fig 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 $mL^{-1}$, Fig 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 (Fig 4). However, after 48 h, reduction in bovicin HC5 activity was observed for all conditions tested, being more pronounced for cultures growing in SAT (Fig 4).

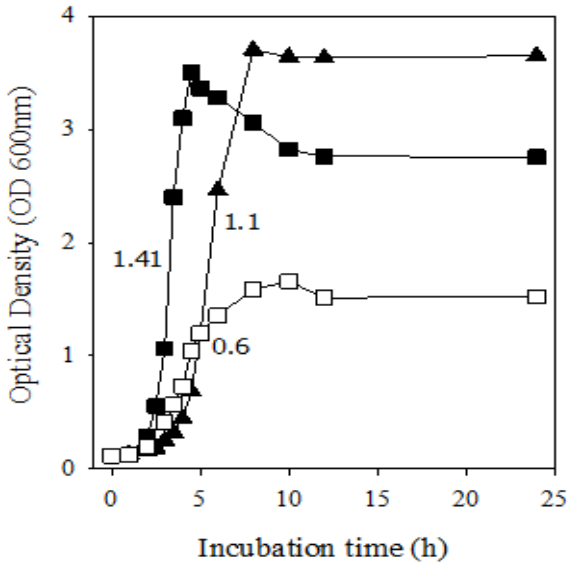


**Figure 3** The effect of growth atmosphere on *S. bovis* HC5 growth. *S. bovis* HC5 was incubated under aerobic condition (open squares), continuous $CO_2$ flow (closed squares) and in sealed anaerobic tubes (closed triangles). The cultures were maintained at 39ºC and the growth was monitored by changes in optical density ($OD_{600\ nm}$). The estimated specific growth rates ($h^{-1}$) for *S. bovis* HC5 are indicated for each growth condition.

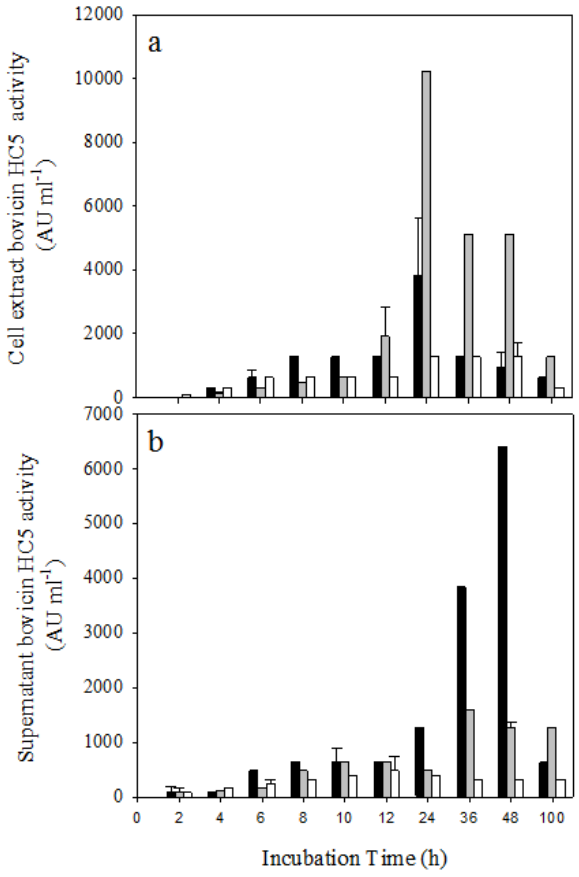


**Figure 4** Production of bovicin HC5 under aerobic conditions (white bars), continuous $CO_2$ flow (black bars) and sealed anaerobic tubes (gray bars). At each time interval, samples were taken and bovicin HC5 activity (AU $mL^{1}$) in the cell extract (a) and in the culture supernatant (b) were determined using *A. acidoterrestris* DSMZ 2498 as the indicator organism.

## DISCUSSION

Lactic acid bacteria are fastidious organisms that often require complex media to sustain the production of biomass and the biosynthesis of metabolites with industrial applications, including antimicrobial peptides **(Kim *et al.*, 2006)**. Previous work demonstrated that strains of *Pediococcus acidilactici* **(Anastasiadou *et al.* 2008)**, *Lactococcus lactis* **(De Vuyst *et al.*, 1996)**, *Micrococcus* sp. GO5 **(Kim *et al*,. 2006)** and *Lactobacillus rhamnosus* **(Aguilar-Uscanga *et al.*, 2013)** grow better and produce more bacteriocin in complex media. To be commercially attractive, bacteriocins should be obtained at high yields, preferentially, in inexpensive media **(Aguilar-Uscanga *et al*., 2013)**.

Several studies have tested alternative or lower cost carbon and nitrogen sources to produce bacteriocins **(Todorov *et al*., 2004; Metsoviti *et al*., 2011; Aguilar-Uscanga *et al*., 2013)**. However, most lactic acid bacteria require complex media to growth and cannot sustain anabolism in the absence of several growth factors, including vitamins and amino acids.

*Streptococcus bovis* HC5 is an unusual lactic acid bacterium that has only few nutritional requirements, being able to use ammonia as the sole nitrogen source **(Wolin *et al*., 1959).** Previous studies also showed that *S. bovis* HC5 can produce high levels of bovicin HC5 using sugar cane juice and cheese whey as the only source of carbon and nitrogen **(Carvalho *et al*., 2009)**.

In this work, *S. bovis* HC5 grew better and produced greater levels of bovicin HC5 in basal media than in complex media (MRS, BHI or M17) generally used to grow lactic acid bacteria. Cultivation of *S. bovis* HC5 in rich media not only reduced overall bacteriocin production, but also induced the secretion of the peptide in the supernatant, a characteristic that did not depend on the presence or absence of oxygen for growth. On the other hand, growth of *S. bovis* HC5 in basal media induced greater bacteriocin production and much more of the peptide remained attached to the cells.

This feature appears ecologically sound to a bacterium isolated from the bovine rumen, an ecosystem where the concentration of free sugars and amino acids is often very low and the competition for nutritional resources is usually very high **(Eijsink *et al*,. 2002; Nigutova *et al*., 2007)**. In order to outcome its competitors in a highly populated environment, it would be advantageous to regulate bacteriocin production according to the growth condition **(Eijsink *et al*., 2002).** In this way, if resources are readily available, more carbon and energy will be diverted to biomass instead of bacteriocin production.

The fact that more bovicin HC5 remains attached to the producer cells is also advantageous for the purification of the peptide, as it can be easily separated from the components of the growth medium, and one chromatographic step can be used to purify the extracts containing bovicin HC5 **(Paiva *et al*., 2011)**. Results obtained by **Mantovani and Russell (2003b)**, **Houlihan *et al*., (2004)** and **Xavier *et al.* (2006)** suggest that cell-associated bovicin HC5 could be more important than cell-free activity, being a critical factor in colonization in the rumen **(Russell and Mantovani, 2002)**.

Besides nutrient availability, other growth conditions such as pH, temperature and incubation atmosphere also have great influence on bacteriocin production by lactic acid bacteria **(Leroy and De Vuyst, 1999; Mataragas *et al*., 2003; El-Shouny *et al*., 2012)**. Temperature and pH not only affect the growth of the producer strain but also interferes with the stability of the peptide during post-translational modification. In addition, temperature and pH can also modify the aggregation, absorption, proteolysis and the activity of the bacteriocin **(Cheigh *et al*., 2002; Drosinos *et al*., 2006).**

*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 when the initial pH was 6.5 and the temperatures varied from 30°C to 39ºC. 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)**.

Our results indicated that initial pH values below and above 6.5 had a negative impact on bovicin HC5 production. The observation that little bacteriocin activity was detected at lower pH values could be explained by the fact that at these pH conditions the growth of *S. bovis* HC5 is impaired, which also limits bacteriocin production (Tab 2). **Houlihan *et al.* (2004)** did not study 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 solution to access bovicin HC5 activity in this study, 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.

To confirm the role of medium pH on bovicin HC5 production, cultures of *S. bovis* HC5 were maintained at constantly pH value during growth in basal media (Tab 3). At this condition, the bovicin HC5 activity at pH 5.5 was at least 100-fold greater than the activity recovered from cultures with only the initial pH adjusted (Tab 2 and 3). In an earlier work, **Mantovani and Russell (2003b)** 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. The increase in specific activity of bovicin HC5 cannot be explained by a greater amount of ionized carboxyl groups in the cell envelope, which could modify the net negative charge of the cell and the binding affinity of the peptide to the producer cells. The decrease in pH increased the activity of bovicin HC5 in the cell-free supernatant, but the cell-attached bacteriocin also showed a dramatic increase in activity, which suggest that the regulation of bacteriocin production was being affected by media pH.

Temperature had only a modest effect on synthesis of microbial biomass and bovicin HC5 production in *S. bovis* HC5. In the range of temperature usually used for growth of *S. bovis* HC5 (39$^{o}$C-41$^{o}$C), the level of bovicin HC5 remained high, indicating that pH, rather than temperature, has a much more pronounced effect on bacteriocin production.

Previous work showed that lactic acid bacteria can grow under microaerophilic to strictly anaerobic condition **(Klein *et al.*, 1998)**. Although the production of many lactic acid bacteriocins has been studied under strict anaerobic conditions, at least to some bacteriocins (e.g. nisin), an oxygen-enriched atmosphere can enhance the production of antimicrobial peptides **(Cabo *et al*., 2001)**. As shown if Fig 2, *S. bovis* HC5 also have the ability to grow and produce bacteriocin under aerobic conditions. However, in the presence of oxygen, the specific growth rate of *S. bovis* HC5 and the levels of cell-free and cell-associated bovicin HC5 were much lower than the anaerobic cultures.

**Neysens and De Vuyst (2005**) showed that amylovorin titers were higher when the producer strain was maintained under 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 reflected in a high specific growth rate and a decreased production of cell-associated bovicin HC5 compared to the cultures maintained in anaerobic sealed tubes.

## CONCLUSION

In conclusion, our results show that culture pH, temperature and incubation atmosphere influence bovicin HC5 production and optimization of the growth conditions is required to improve the activity recovered from *S. bovis* HC5 cells. Bacteriocin production was favored under conditions of controlled pH (5.5), anaerobiosis and incubation temperature of 39 °C. Based on the ability of *S. bovis* HC5 to grow in simple media and produce large amounts of bovicin HC5, further work will be needed to obtain bovicin HC5 at high yields and lower costs, compared to other bacteriocins produced by fastidious lactic acid bacteria.

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