TÉCNICO LISBOA

Effect of cultivation conditions on antimicrobial production in *Lactococcus lactis*

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"You can have results or excuses. Not both."

Abstract

The awareness of the adverse effects of chemical preservatives has been increasing over the years and so, the demand to improve current methods for food preservation has become higher, together with the request for more “natural” food. Therefore, more attention is being brought to the antimicrobial compounds produced by lactic acid bacteria: gram-positive, facultative anaerobic bacteria with a fermentative metabolism that are safe to consume and widely used in industry.

The aim of this project was to determine the effect of cultivation conditions on antimicrobial production in *Lactococcus lactis*. Fermentation experiments in 0.5L fermenters confirmed that antimicrobial production follows primary metabolite kinetics and that the end of the exponential phase is the best time to harvest the broth containing the antimicrobial. In several experiments, the effect of varying the substrate concentration, the pH, the medium composition and other fermentation conditions was established. This work resulted in the design of optimal cultivation conditions with respect to fermentation time and medium costs with a maximized yield of antimicrobial compound production.

Keywords: fermentation, antimicrobials, yield optimization, lactic acid bacteria

Resumo

O alerta para os efeitos adversos dos conservantes químicos tem vindo a aumentar ao longo dos anos, bem como a procura para aperfeiçoar os atuais métodos de conservação alimentar, reforçando o interesse em alimentos naturais e biológicos. Assim, tem vindo a aumentar atenção para os compostos antimicrobianos produzidos por Bactérias Ácido-Lácticas: bactérias gram-positivas, anaeróbicas facultativas com um metabolismo fermentativo, seguras para o consumo e vastamente utilizadas na indústria.

O objectivo deste projeto foi determinar os efeitos das condições de cultura de *Lactococcus lactis* para a produção de um composto antimicrobiano. Ensaios de fermentação realizados em fermentadores de 0.5L confirmaram que a produção do composto antimicrobiano segue uma cinética de metabolito primário e que a melhor altura para recolher o meio de fermentação que contém o composto antimicrobiano é o final da fase exponencial. O efeito da variação da concentração do substrato, do pH, da composição do meio e de outras condições de fermentação ficou estabelecido após vários ensaios. Este trabalho resultou no design de condições óptimas de cultura, no que diz respeito ao tempo de fermentação e aos custos do meio de cultivo com um rendimento máximo da produção do composto antimicrobiano.

Palavras-chave: fermentação, composto antimicrobiano, optimização do rendimento, bactérias ácido-lácticas

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List of abbreviations

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
$CaCl_2$	Calcium Chloride
$CaCO_3$	Calcium Carbonate
CM	Complex Medium
Dha	Didehydroalanine
Dhb	Didehydrobutyrine
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drugs Admnistration
GRAS	Generally Recognized As Safe
IU	International unit
LAB	Lactic acid bacteria
Lan	Lanthionine
MF	Multifors
NAD +	Nicotinamide adenine dinucleotice
NADH	Nicotinamide adenine dinucleotice - hydrogen
NaOH	Sodium Hydroxid
OD	Optical density
Qv	Volumetric productivity in g L^{-1} h^{-1}
Rpm	Rotation per minute
w/w	Weight/weight

w/v	Weight/volume
YEP	Yeast Extract Paste
β- MeLan	β-methyllanthionine

1. Introduction

This study aims to efficiently produce a compound A ferment to be used as a natural preservative in food. In the first part of this project (chapter 4.1, 4.2, 4.3, 4.4), the aim was to test the effects of varying substrate concentration, pH and medium composition on the growth and biomass production of *Lactococcus lactis* and, consequently, on compound A activity. Based on earlier in house studies and literature research, 0.5 L fermentations were performed and the optical density, lactate and sucrose concentration, and compound A activity were measured. These results were used to design the optimal medium composition for high compound A production.

The second part of the project (chapter 4.5) can't be published and is presented in a separate confidential appendix.

In the last section of this work (chapter 5), the main conclusions are presented, together with some recommendations for future investigation on the effect of cultivation conditions on antimicrobial production in *Lactococcus lactis*.

2. Theoretical background

2.1 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are described as gram-positive, facultative anaerobic bacteria with a fermentative metabolism (incomplete Krebs cycle). They are industrially important organisms recognized as safe to consume, with health and nutritional benefits. The antimicrobial activities of LAB have long been known and traditionally applied, playing an important role in food fermentations, food preservation and intestinal ecology.[1], [2]

Lactic acid bacteria's long history of application in fermented foods is due to their beneficial influence on nutritional, organoleptic (such as taste and texture), and shelf-life characteristics. They are capable of inhibiting various microorganisms in a food environment and it has been shown that some strains of LAB possess interesting health-promoting properties against some pathogens like *Escherichia coli* and *Salmonella*, for example. *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* are some examples of the well-studied genera of LAB. [1], [3]

In this sense, LAB display a wide range of antimicrobial activities, causing rapid acidification of the raw material through the production of organic acids such as lactic acid (the most important one) and acetic acid. However, certain strains of LAB are further known to produce bioactive molecules like ethanol, carbon dioxide, flavor compounds (e.g. diacetyl, acetoin and acetaldehyde), formic acid, fatty acids, hydrogen peroxide, some enzymes and also antimicrobial proteins called bacteriocins. [1], [3]

2.2 Bacteriocins

Nowadays, the awareness of the adverse effects of chemical preservatives, such as salts and antibiotics, is increasing and there is a higher demand to improve the current methods for food preservation and safety. The request for more "natural" food brings more attention to antimicrobial compounds produced by bacteria from both the food industry and scientists. [4]

Therefore, the bacteriocins produced by LAB are very useful in food protection for several reasons. In the first place, they are small ribosomally synthesized antimicrobial peptides or proteins that can be modified by genetic engineering. Secondly, by being natural substances they are biodegradable and finally, because each different bacteriocin has its own unique and rather narrow killing spectrum, thus allowing manipulation of food microbial ecosystems. [3], [4]

The antibacterial spectrum frequently includes closely related Gram-positive spoilage organisms and food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*. Besides their antimicrobial action towards undesirable bacteria, bacteriocins are believed to contribute also to the competitiveness of the producer cells. On the other hand, the activity against Gram-negative bacteria such as *E. coli* and *Salmonella* has been shown, but usually only when the integrity of the outer membrane has been compromised, for example, after osmotic shock, low pH treatment or in the presence of a detergent or chelating agent.[3]

Among bacteriocins from LAB, distinction can be made between structure and function dividing them in three classes, presented in table 2.1.

Table 2. 1 - Classes of bacteriocins and its properties.[3]

Class	Name	Properties	Special features
I	Lantibiotics	- Small - Heat-stable - Lanthionine-containing - Single and two-peptide bacteriocins	Inactive prepeptides subject to extensive post-translational modification
II	Peptide bacteriocins	- Small - Heat-stable - Non-lanthionine-containing	IIa – pediocin-like or *Listeria*-active bacteriocins IIb – two-peptide bacteriocins IIc – circular bacteriocins
III	Bacteriolysins	- Large - Heat-labile - Lytic proteins	-

Extensive efforts have been made to resolve the relationship between structure and function for both class I and class II bacteriocins. The majority of the class I and class II bacteriocins are active in the nanomolar range, causing membrane permeabilization and leading to the dissipation of membrane potential and the leakage of ions, ATP, and other vital molecules from the target bacteria. [3]

2.3 Lantibiotics

The lantibiotics are a group of polycyclic LAB bacteriocins, containing 19-34 amino acids, which are characterized by the occurrence of intra-chain sulphur bridges and unusual thioether amino acids, such as lanthionine (Lan) and β-methyllanthionine (β- MeLan). The lantibiotics also often possess α, β-unsaturated amino acids, for instance didehydroalanine (Dha) and didehydrobutyrine (Dhb), whose structure is presented in figure 2.1. These unusual amino acids result from post-translational enzymatic modifications of inactive prepeptides and can be divided in two separate groups according to their structure and mode of action: the compound A-like (type A) and to the duramycin-like (type B). The type A lantibiotics are cationic, linear and screwed in shape, and they inhibit the growth of bacteria by depolarizing the cell membranes of target cells. On the other hand, the type B lantibiotics are globular molecules and have a low negative net charge. [1],[4]

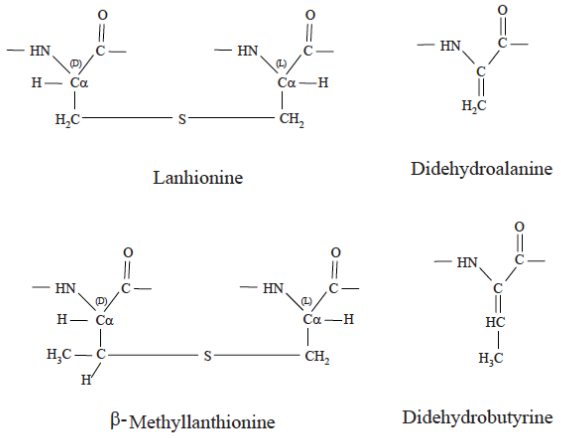

Figure 2. 1 - Structure of the four unusual thioether amino acids that are presente in lantibiotics: Lanthionine, Didehydroalanine, β-methyllanthionine and Didehydrobutyrine. [4]

2.4 Compound A

2.4.1 Structure and properties

To date, approximately 50 lantibiotics are known, and their number continues to grow. A very well known representative of type A lantibiotics is compound A and its production is the object of this study.

Compound A is the most extensively studied and characterized antimicrobial protein produced by lactic acid bacteria. It contains 34 amino acids and 4 unusual amino acids (dehydroalanine, dehydrobutyrine, lanthionine and β-methyllanthionine), presented in figure 2.2. These unusual amino acids should be responsible for the functionally important properties of compound A molecule, i.e. thermo stability and bactericidal action. [1],[5],[6]

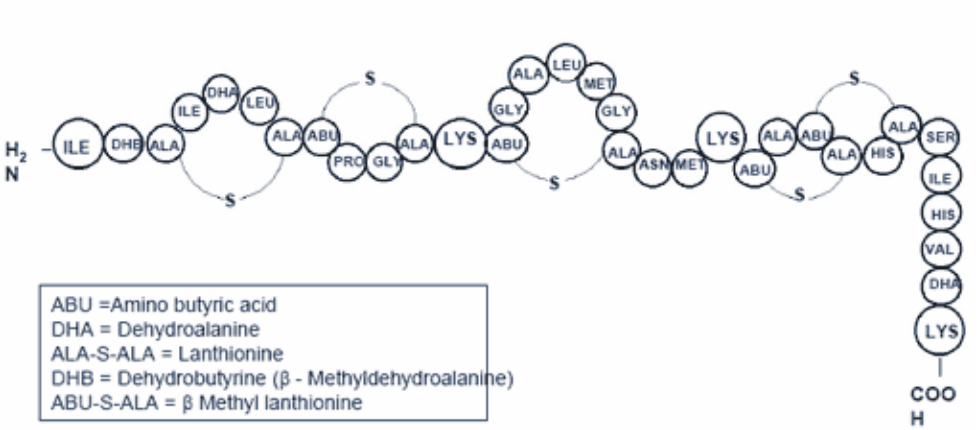

Figure 2. 2 - Amino acid composition of compound A.[7]

This protein is heat stable and insoluble in nonpolar solvents. Its solubility in polar solvents is pH dependent (12% at pH 2.5, 4% at pH 5), meaning it increases when the pH is lowered. Its isoelectric point is on the alkaline side.

Compound A is most stable at low pH and can even stand sterilization (121 ^{o}C for 20 min) when at pH 2.5. However, in a pH above 7, the molecules undergo irreversible changes with loss of activity.[5]

The biosynthesis of compound A occurs in two steps: a precursor peptide is synthesized ribosomally followed by post-translational enzymatic modifications converting the inactive precursor into a mature bioactive peptide. These modifications include dehydration of specific prepeptide serine and threonine residues, with subsequent addition of neighboring cysteine sulphur to the double bonds of the didehydroamino acids resulting in the formation of thioether bridges. [15]

There are five naturally occurring compound A variants (A, Z, Q, F and U), which differ from each other by amino acid composition. The strains used in this work produce the bacteriocin in the A and Z variants, that can be distinguished by a single amino acid change. Concerning its properties, bacteriocin Z appears to be slightly more diffusable in agar and more soluble in neutral pH. [23]

2.4.2 Fermentative metabolism

Nowadays, compound A is commercially produced exclusively by homolactic fermentation of some *Lactococcus lactis* strains. This bacteria consists of three subspecies: *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *hordniae*. The first two subspecies are widely used in industry as starter cultures for the production of cheese, butter and buttermilk. [21]

Lactococcus lactis strains are mesophilic and homo fermentative, which mean they grow optimally at temperatures around 30°C and they produce predominantly L-lactic acid from pyruvate via the NAD-dependent lactate dehydrogenase (LDH).

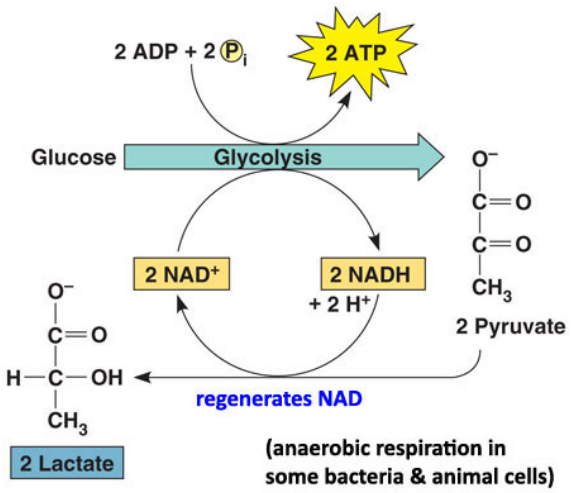

Figure 2. 3 - Schematic overview of the anaerobic fermentative pathway in *Lactococcus lactis*.[22]

This LAB have a specific phosphoenolpyruvate-dependent carbohydrate phosphortransferase system (PEP:PTS) to simultaneously take up and phosphorylate carbohydrates that, after uptake, are hydrolyzed to glucose. Afterwards, the glucose is phosphorylated and further metabolized to pyruvate via glycolysis. During anaerobic fermentation, the NADH formed during the glycolysis is reoxidized to NAD+ by the reduction of pyruvate to lactate. Overall, one molecule of glucose (or any six-carbon sugar) is converted to two molecules of lactic acid: $C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOOH$. A simplified scheme of this fermentation metabolism is presented in figure 2.4. [21], [16]

Studies have been made concerning the moment when compound A is formed in fermentation. Some researchers affirm that since the bacteriocin production does not begin until the early stages of growth and increases sharply after considerable cell growth has occurred, it seems to be synthesized as a secondary metabolite (= organic compound that is not directly involved in the normal growth, development, or reproduction of organisms). [5]

However, other studies state that the observations of compound A do not match those of secondary metabolites, because the bacteriocin production stops when biomass growth enters the stationary phase. They also observed a decrease of biomass concentration in the stationary phase and assume that this decrease is not caused by a high concentration of the bacteriocin within the cells. Instead, they consider that some *Lactococcus* strains might have certain lysogenic potential and that the cell lysis proteases are released into the medium, possibly degrading compound A.[1]

Therefore, compound A is known to follow primary metabolite kinetics, being growth dependent. Besides biomass and compound A, the major product formed from the carbohydrate source is lactic acid, which is the reason for the need of a pH control or buffering of fermentations. [5]

2.4.3 Activity and mode of action

Compound A is known to strongly inhibit the growth of food borne pathogens like *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus* and several other gram-positive bacterial species. However, it is not active against molds, yeasts and gram-negative bacteria, unless the outer membrane is first destabilized. [23]

The effect of compound A on the targeted bacteria is exerted in the cytoplasmic membrane. The main activity appears to be the formation of pores in the lipid bilayers of the bacterial membrane that causes an increase in membrane permeability with a concomitant leakage of ions, ATP, and amino acids, which lead to depolarization and dissipation of the bacterial membrane potential. The combined effect of energy depletion and the efflux of essential compounds inhibit the synthesis of macromolecules and results in an ultimate cell death accompanied, in most cases, by cell lysis. On the other hand, the

action of compound A against spores is caused by binding protein residues with sulfydryl groups on the spore coats resulting in mechanical rupture.[23], [5]

2.4.4 Toxicity and application

The fact that compound A is produced by lactococci, which occur naturally in raw milk and cheese, is an indication of its harmless nature: humans and animals have ingested it over the past centuries, without apparent illness effects. While this does not rule out the possibility of adverse effects, it does indicate that the bacteriocin has low toxicity levels, if any at all. However, it was intensely studied all over the world and the LD_{50} value was found to be similar to that of common salt (about $7g.kg^{-1}$ body weight). Furthermore, the consumption of compound A-containing products would not result in any alteration of the intestinal bacterial flora because enzymes of the intestinal tract inactivate this bacteriocin. [24]

Therefore, compound A is the only bacteriocin accepted in more than 50 countries as a safe and natural food preservative. The Food and Drug Administration (FDA) approved it in 1988 as Generally Recognized as Safe (GRAS), meeting the demands for natural food with fewer chemical additives. [23]

As a natural food preservative, compound A is mostly used in processed cheese and cheese spreads, in canned vegetables and fruits, in sterilized milk, milk drinks and, nowadays, in meat. With this vast food applications, it is considered to be one of the most promising and important group of natural inhibitors, with a highly economic added value.[23], [24]

As the lactic acid bacteria are fastidious microorganisms they require a complex medium to grow and to produce the bacteriocin, making the purification of the latter rather difficult. Furthermore, many bacteriocin molecules tend to associate with other molecular substances present in the culture medium, display a high degree of hydrophobicity and form protein aggregates. This fact makes the downstream processing more difficult, requiring the design of specific purification protocols for each bacteriocin. Therefore, the impact of different parameters such as the producer strain, composition of medium, temperature, pH and aeration have to be studied in order to formulate an effective process design for compound A production with a medium composition as simple and cheap as possible. [15], [16]

3. Materials & Methods

3.1 Bacterial strain

The bacteriocin-producing strains (*Lactococcus lactis*) used in this study are listed in table 3.1.

Table 3. 1 – Strains used in this project in order to obtain the highest compound A production, by fermentation.

Working name	Compound A variant	Adaptation
Strain 1	Z	9x 15000 IU
Strain 2	A	17x 20000 IU

The public compound A production strain (Strain 2) used has its origin on *Lactococcus lactis* subsp. *lactis* ATCC 11454 which was improved and adapted to grow in the presence of compound A concentrations of 20000 $IU.g^{-1}$ and the second strain listed (Strain 1) was improved and grown in the presence of 15000 $IU.g^{-1}$, resulting in strains with a higher bacteriocin A and bacteriocin Z production, respectively.

Both strains were grown in shake flasks with 100 mL of the following medium: 2% w/w sucrose, 1% w/w YEP (50% ds.) and 1% w/w $CaCO_3$ that constituted the stock cultures to be used in this work. 1.5 mL of the stationary phase cultures together with 0.5 mL of 60% v/v kosher glycerol were put in aliquots, labeled and then stored at -80^{o}C.

3.2 Inoculum

Before every experiment, a 40 mL pre-culture with 2% w/w sucrose, 1% w/w YEP (50% ds.) and 1% w/w $CaCO_3$ was inoculated with 0.3 mL of the -80^{o}C stock cultures. The flask was then incubated for approximately 10 to 15h, at 30^{o}C, with some agitation to prevent the precipitation of $CaCO_3$. By measuring the optical density at 600 nm it was possible to determine the growth of the bacteria.

3.3 Media and Fermentation Conditions

The anaerobic fermentations were run in 0.5 L Multifors fermenters (Infors HT, Switzerland) using the standard protocol described in Appendix 1. The standard medium composition being used before this study was: 4% w/w sucrose, 2% w/w YEP (50% ds.) from Biospringer and 0.5 $g.L^{-1}$ $CaCl_2.2H_2O$ from Acros. The fermentations were performed at 30^{o}C, with a stirring speed of 250 rpm (to keep the fermentation broth homogeneous) and a controlled pH of 5.5, measured by a pH electrode (Mettler Toledo, USA) and maintained by the addition of a titrant: 20% NaOH.

To test the effect of the medium composition, a Complex Medium (CM) suggested by De Vuyst was used and had the following composition: 3% w/w sucrose, 1% w/w YEP (50% ds.) from Biospringer, 1% w/w peptone, 1% w/w KH_2PO_4, 2 $g.L^{-1}$ NaCl and 0.2 $g.L^{-1}$ $MgSO_4.7H_2O$.[1]

The anaerobic fermentations were performed as batch fermentations operated with no aeration and the reactors were inoculated with 2.5% w/w of the Strain 1 pre-culture.

Before starting any fermentation, the reactors were sterilized at 121^{o}C for 20 minutes. Regular samples were taken and analyzed for sucrose and lactate concentration, optical density at 600nm and compound A activity.

3.4 Analytical methods

The optical density of the cells was determined using a spectrophotometer at 600 nm. The samples were diluted with an EDTA solution (pH8, 50mM), depending on the expected density, and measured against the fermentation medium without cells, as a blank.

To determine the concentration of sucrose and lactate in the fermentation broth, a biochemical analyzer (SalmenKipp YSI 2700: Glucose and Lactate analyzer) was used. 1 mL of the fermentation broth was centrifuged at 12500 rpm for 2.5 minutes and the supernatant was diluted with demineralized water so the values were within the detection limits of the YSI (0 – 0.5 g.L^{-1} for lactate and below 2.5 g.L^{-1} for glucose). Therefore, to measure the lactate concentration and sucrose concentration, a 1:100 (v/v) dilution and a 1:10 (v/v) dilution were made, respectively.

However, since the YSI only measures glucose, the sucrose solution had to be converted, incubating it for 30 minutes at 55^{o}C in the presence of 20µL of the enzyme invertase.

3.5 Compound A activity bioassay

A Sigma Standard Compound A 1000000 IU.g^{-1} (variant A) was used to prepare the bacteriocin standard solutions, ranging in activity from 30 to 600 IU.g^{-1} in order to set a calibration curve for compound A activity. Afterwards, the fermentation samples of interest were diluted with 0.02M HCl to fit into the standard range and a few drops of 5M HCl were used to adjust their pH between 2.0 - 2.5.

Posteriorly, the standards and the fermentation diluted samples were boiled for 15 minutes in a water bath at 100ºC, in order to be extracted, and the BHI/agar plates were prepared and inoculated with *Lactococcus lactis* subsp. *cremoris* and some wells were crafted and properly marked.

Finally, the samples were loaded in the wells and incubated overnight at 30^{o}C. The inhibition of growth was determined measuring the diameter of the circular halo, using a digital caliper, and the compound A concentration of the samples was determined using the standard calibration curve and the dilution factors. However, it's necessary to take into consideration that this analysis may contain some errors (10-15%) within the actual compound A activity since the diameter of the halo is measured optically and it was performed for more than one analyst.

This analysis was performed by the Analytical support team and the complete protocol for compound A activity analysis using an agar diffusion method is described in the Appendix 3.

4. Results and Discussion

4.1 Production of compound A during growth

Several studies demonstrate *L. lactis* fermentation profiles which indicated that compound A production shows primary metabolite kinetics: the pre-bacteriocin is synthesized early in the active growth phase and compound A production rate is maximal towards the end of the same phase coming to a complete halt when the cells enter the stationary phase. However, the fact that compound A production increased rapidly only at the end of the exponential growth phase, have been raising some controversy whether it follows secondary metabolite kinetics instead. More studies were made concerning the expression of this bacteriocin precursor gene that showed that compound A activity could be detected early in the exponential growth phase and that the increase of compound A production rate towards the end of the active growth phase could be due to the delayed formation of the necessary pre-bacteriocin modifying genes, reaffirming that compound A production displays primary metabolic kinetics. [1], [15]

In this experiment, compound A production kinetics of *Lactococcus lactis* was examined with the standard fermentation conditions described in table 4.1, following the fermentation protocol presented in Appendix 1. These were the conditions that were being used before the initiation of this work. However, due to the decision made in point 4.2 the following results are based on the average of two reference fermentations performed with 3% of sucrose, instead of 4%.

Table 4. 1 - Standard fermentation conditions that were being used before the initiation of this study.

Medium	4% sucrose 2% YEP (50% ds.) 0.5 $g.L^{-1}$ $CaCl_2$
pH	5.5
Temperature	30^{o}C
Agitation	250 rpm
Base titrant	NaOH
Bacterial strain	Strain 1

The batch fermentation profile of microbial growth and compound A production at a controlled pH is presented in Figure 4.1. The exponential growth phase took place during a period of about 5-6h at a maximum specific growth rate of 0.41 h^{-1} and a doubling time of 1.69 h.[17] The maximum cell density was reached after 7-8h and the compound A production levels appear to follow a parallel tendency: the higher titres were reached at the end of the exponential phase and corresponded to the maximal biomass. Consequently, it shows that the compound A was produced in the active growth phase, confirming the literature results that this bacteriocin production follows primary metabolic kinetics.

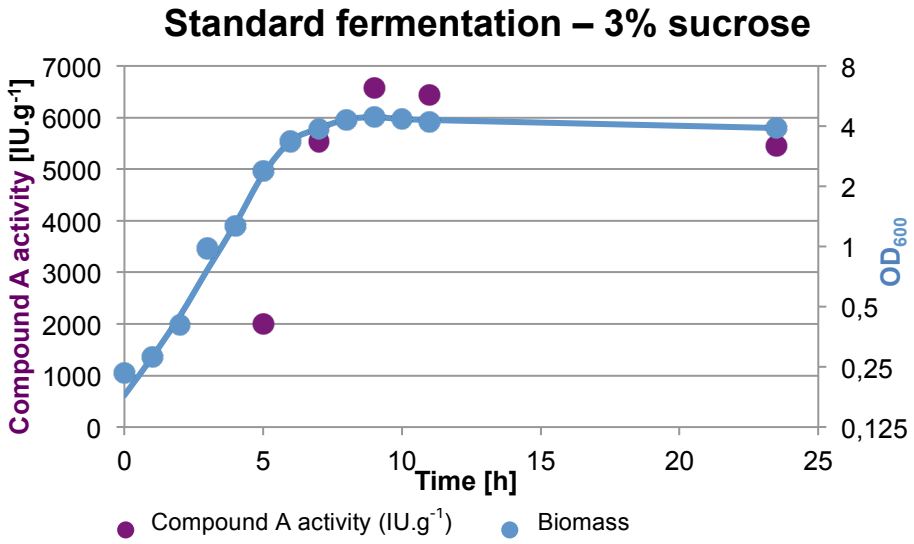

Figure 4. 1 – Compound A activity and biomass values of the standard 3% sucrose fermentations of strain 1.

After reaching peak values, sometimes the biomass and compound A levels decreased with a prolonged fermentation time. The decrease of the bacteriocin titre after 8h of fermentation implies that the fermentation process would need to be stopped by then, making this the best time to harvest the broth, in order to achieve a maximum compound A productivity.

A dramatic decrease in compound A level after reaching the peak value is suspected to be a result of proteolytic degradation and/or adsorption of the bacteriocin by producer cells, as stated in literature. In this sense the best method to decrease the loss of compound A is to recover the product during fermentation and protect it at low pH as soon as possible. [16]

4.2 Effects of varying sucrose concentration

To determine the effect of varying the carbon source concentration, several batch experiments in 0.5L Multifors fermenters were performed with 1%, 2%, 3% and 4% of sucrose, using the Strain 1 (protocol in Appendix 1). The rest of the conditions were kept the same, as in the standard fermentation (30°C; pH 5.5; 250 rpm; 2%YEP, 0,5 $g.L^{-1}$ $CaCl_2$; base: NaOH).

In figures 4.2 to 4.5 it is possible to observe the biomass formation during the fermentation time, measured by optical density, for the 4 sucrose concentrations tested. It has to be taken in account that only one experiment was performed with 1% of sucrose, two were performed with 2%, four experiments with 3% of the carbon source and also two with 4% of sucrose, which means that more experiments should be done to reaffirm this results.

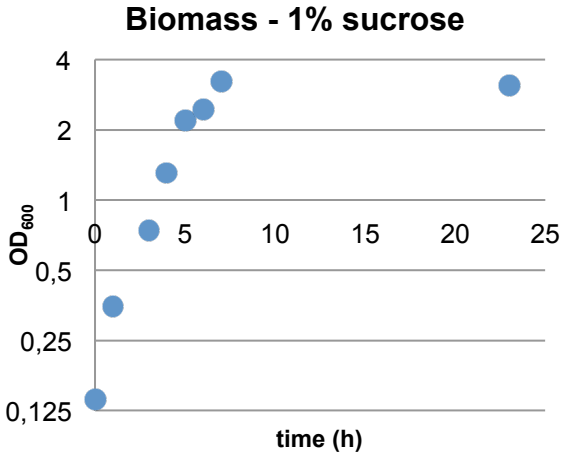

Figure 4. 2 - Growth curve of Strain 1 with the standard fermentation conditions but with 1% sucrose.

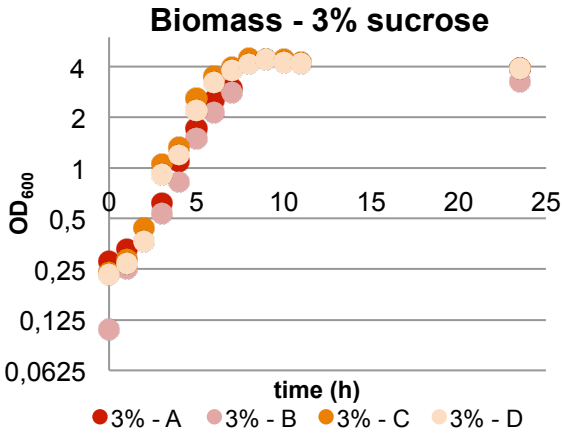

Figure 4. 4 - Growth curve of Strain 1 with the standard fermentation conditions but with 3% sucrose. A, B, C and D correspond to four different experiments.

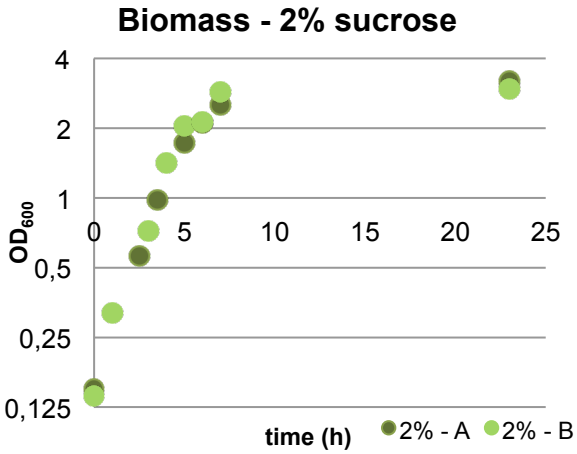

Figure 4. 3 - Growth curve of Strain 1 with the standard fermentation conditions but with 2% sucrose. A and B correspond to two different experiments.

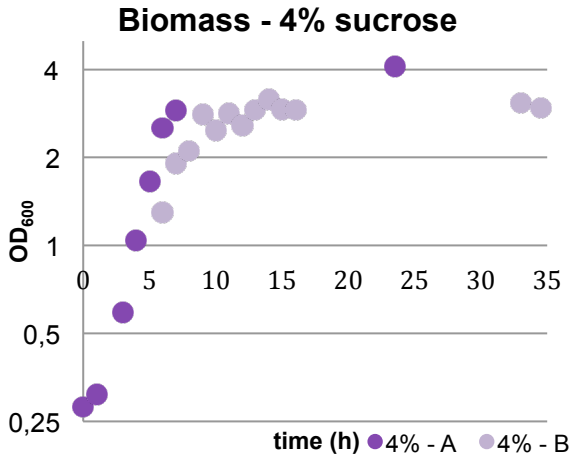

Figure 4. 5 - Growth curve of Strain 1 with the standard fermentation conditions but with 4% sucrose. A and B correspond to two different experiments.

In theory, if we provide a higher concentration of substrate, the cells should also show a higher growth, since there is a bigger amount of the carbon source to be converted. However, as it is possible to observe by the previous graphs, biomass has a very similar growth curve in all four sucrose concentrations, indicating that probably the ATP generated in the higher sucrose concentrations is being used for maintenance, instead of cell growth, or that something else is contributing to the high OD_{600} values that are shown with 1% and 2% of sucrose.

It is shown in literature that the carbon source provides energy for the logarithmic-phase growth and that amino acids such as arginine, for example, provide energy after carbohydrate exhaustion, increasing the survival time, the cell numbers and ATP generation. This fact may explain the higher biomass values observed in fermentations with low sucrose concentrations, in which the carbon source is rapidly depleted.[8]

However, to decide which is the best sucrose concentration it is necessary to take into account several other factors such as the fermentation time, the tailing effect, the compound A activity values and also the fermentation costs.

The following table comprises the biomass values, sucrose and lactate concentration and compound A activity values for each carbon source concentration. These values represent an average of the fermentations performed with each sucrose concentration and concern the end of the fermentation time. The fermentation profiles of every experiment done to test the effect of varying the substrate concentration can de found in Appendix 4.

Table 4. 2 - Average of the results (end of fermentation time) of the anaerobic fermentations performed with the standard conditions, varying the substrate concentration in 1, 2, 3 and 4% of sucrose.

	1% sucrose	**2% sucrose**	**3% sucrose**	**4% sucrose**
Biomass (OD_{600})	3.11	3.07	3.76	4.09
Sucrose concentration ($g.L^{-1}$)	0	0.26	0.63	4.9
Lactate concentration ($g.L^{-1}$)	8.7	18.8	27	33.5
Compound A activity ($IU.g^{-1}$)	2900	4080	6042	6450

As expected, compound A activity values and the lactate values are higher according to the increase of the carbon source, since this bacteriocin is a primary metabolite and its concentration is dependent on the growth performance of the bacteria. Therefore, the higher compound A activity levels can be found when 3% and 4% of sucrose are used: 6042 $IU.g^{-1}$ and 6450 $IU.g^{-1}$, respectively.

Finally, by looking at figures 4.6 and 4.7, it is possible to better compare these two sucrose concentrations. The fermentation profiles shown concern the average of all the experiments performed with 3% and 4% of the carbon source.

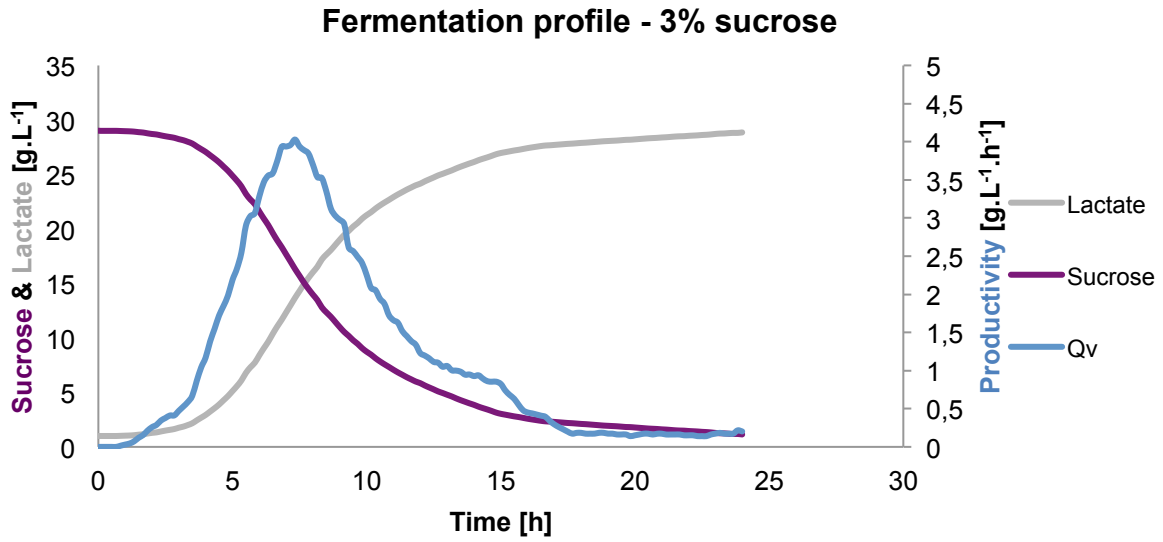

Figure 4. 6 - Average of the fermentation profiles of all experiments performed with the standard conditions and 3% of sucrose.

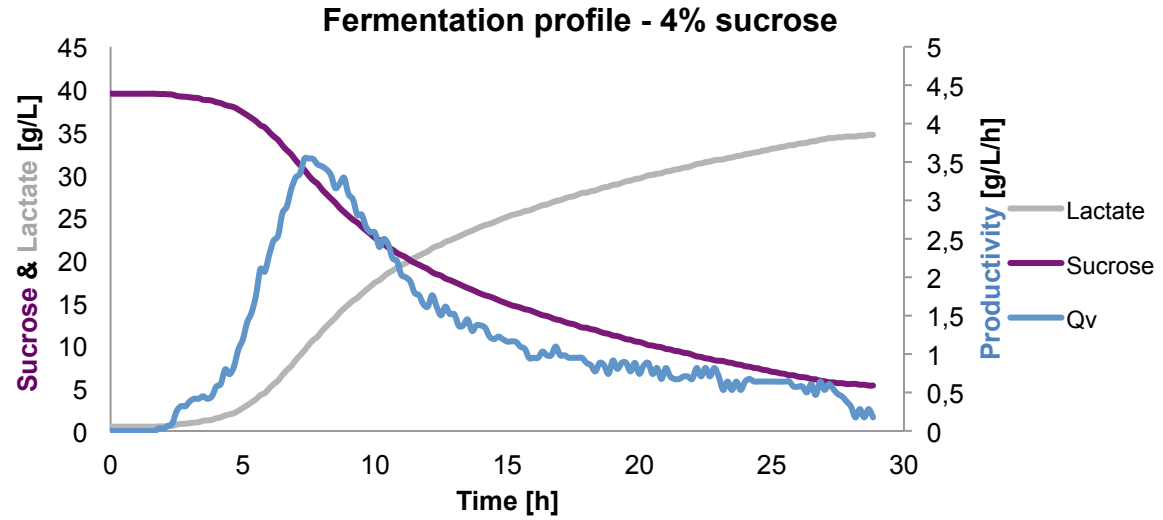

Figure 4. 7 - Average of the fermentation profiles of all experiments performed with the standard conditions and 4% of sucrose.

The Qv ($g.L^{-1}.h^{-1}$) value is a measure of volumetric productivity that is automatically calculated by the fermenter's program with the amount of base that is being added during the fermentation time. The fermentation time is then considered to be the time before all substrate concentration is converted and it can be visible when the Qv reaches zero, which means that no more substrate is being transformed into lactate, excluding the need to add more base to neutralize the fermentation.

Although the productivity values are very similar with both carbon source concentrations, it is possible to observe that the fermentation time is much lower, when using a 3% sucrose concentration. It undergoes a reduction from around 30h (4% sucrose) to a fermentation that runs between 15h and 20h (3% sucrose).

The tailing effect is an effect that occurs when the substrate is not completely consumed but at the same time it is also being converted to lactate at a very slow rate, prolonging the fermentation time. It can be described as a slow decrease in productivity (Qv) and is very visible in the fermentations with 4% sucrose, making it another disadvantage of using these higher concentrations of substrate.

Moreover, using more quantity of sucrose means an increase in the medium costs and, since the fermentations are longer with higher concentrations, the operational costs will be also higher.

For all of the previous reasons, the sucrose concentration that better fits this type of anaerobic fermentations was found to be 3% of sucrose and so this was the substrate concentration taken as a reference for the following experiments performed in this study (Table 4.3).

Reference fermentation conditions:

Table 4. 3 - Reference anaerobic fermentation conditions that were decided to be used from this moment on, in the research.

Medium	3% sucrose 2% YEP (50% ds.) 0.5 $g.L^{-1}$ $CaCl_2$
pH	5.5
Temperature	30^{o}C
Agitation	250 rpm
Base titrant	NaOH
Bacterial strain	Strain 1

4.3 Effects of varying the pH

In theory, since compound A is a primary metabolite its concentration will be higher when the biomass formation is also higher. Other than the influence of the substrate on biomass production and, consequently, on compound A production, there are other parameters that also play an important role. Therefore, the second parameter tested was the pH and its influence on this fermentation process.

It is stated in literature that the optimal growth of *L. lactis* is carried out with a controlled constant pH between pH 5.5 – 6.8, depending on the producer strain.[1] However, some studies were made testing different pH profiles, in addition to the constant controlled pH fermentations, that showed an enhanced compound A production. [9]

It has been shown that at pH 6.8 (controlled fermentation) more than 80% of the compound A synthetized was bound to the cells, whereas at pH below 6, more than 80% of the same bacteriocin was in the culture fluid. Another study reported that the optimal pH for the bacteriocin adsorption to the cells ranges from pH 5.5 to 6.5. Therefore, it was proved that the increase of the pH from 5.5 to 6.8 enhances the adsorption of compound A to the cells but decreases drastically its solubility and stability. [10], [11]

To confirm these results, some experiments were set with the reference conditions (protocol in Appendix 1) but changing the control of the pH to 5, 6 and 7, by the addition of 20% NaOH. The respective biomass formation (measured by the optical density at 600 nm) during the fermentation time is shown in figure 4.8. The adsorption of compound A to the cells was not tested in this work but some conclusions can be taken concerning cell growth and the bacteriocin production. All of the fermentation profiles can be found in Appendix 4.

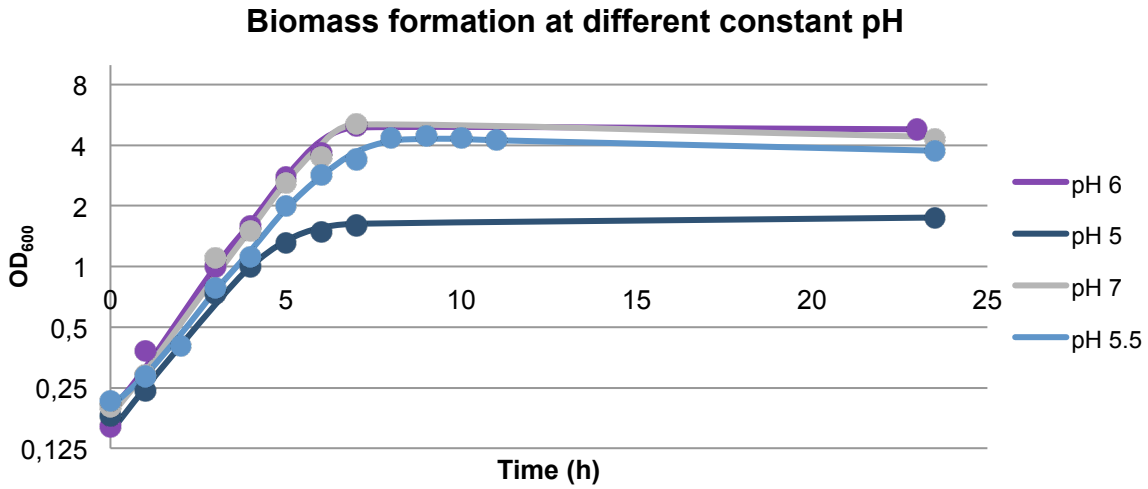

Figure 4. 8 - Growth curve of Strain 1 with the reference fermentation conditions (3% sucrose), performed with a controlled pH of 5, 5.5, 6 and 7.

Observing the graph, it is clear that the cell growth at pH 5 is significantly lower comparing to biomass formation in higher pH fermentations. This may be due to the fact that LAB growth can be inhibited at low pH.

According to the previous results, the cellular growth is very similar when using pH 5.5 (reference), 6 and 7. However, since compound A titres seem to be proportionally related to the biomass yield, especially under pH-controlled conditions, this bacteriocin's activity values have also to be taken into consideration and can be found in table 4.4. Unfortunately, there was an error with the compound A activity analysis and there are no values after 5h and 7h of the fermentation time, at pH 6.

Table 4. 4 – Compound A activity values, determined by the standard compound A bioassay protocol (Appendix 3), of the anaerobic fermentations performed with the reference conditions with a controlled pH of 5, 5.5, 6 and 7, after 5h and 7h of the fermentation time and at its end (23.5h).

	Compound A activity ($IU.g^{-1}$)			
Time (h)	**pH 5**	**pH 5.5**	**pH 6**	**pH 7**
5	710	1623	-	1570
7	1370	4907	-	5340
23,5	920	6043	6600	560

The low compound A activity values at pH 5 can confirm the statement that compound A production is dependent on the biomass formation, showing a primary metabolite behavior.

On the other hand, at pH 7 compound A activity values are also low, and they significantly decrease in the end of the fermentation (23.5h) what can confirm the literature statements: at a higher pH more than 80% of the bacteriocin is bound to the cells. Therefore, compound A concentration on the fermentation broth is lower as confirmed by the compound A activity bioassay values.

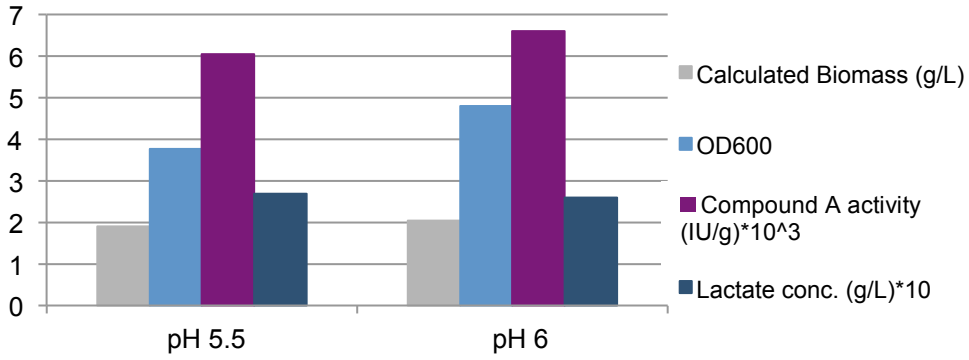

Figure 4. 9 - Comparison of the biomass (measured and calculated), Compound A activity and lactate concentration results (end of fermentation time) on the anaerobic fermentations performed with the standard conditions and at a controlled pH of 5.5 and 6.

Finally, to better compare the controlled fermentation results at pH 5.5 and 6 and to decide which one is the optimal pH to be used in further experiments, figure 4.9 can be observed.

In this figure, it is possible to compare the value of the calculated biomass (formula can be found in Appendix 5) according to the amount of base added, the biomass formation measured by optical density at 600 nm, compound A activity values and the lactate concentration. All of these values concern the end of the fermentation time.

Since the results are very similar, it is safe to say that for this *L.lactis* strain (Strain 1) the optimal constant pH would be between 5.5 and 6. However, the experiments should be repeated and performed with more detail (pH 5.6, 5.7, 5.8 and 5.9) in order to find a more reliable optimal value. Nonetheless, further experiments in this study were taken keeping the pH at 5.5.

Just as a note, two other experiments were designed using the following pH profiles: 1) pH control off after 8h allowing the pH to drop freely via autoacidification (set point pH 6)– the purpose would be to prevent the adsorption of compound A onto the producer cells, as this process is less pronounced in an acidic environment – and 2) pH control off for 10 min in every two hours (set point pH 6) – higher pH is more beneficial for cell growth whereas the pH drops would allow compound A to be released to the fermentation broth.

Table 4. 5 – Biomass and compound A activity values, determined by the standard compound A bioassay protocol (Appendix 3), of the anaerobic fermentations performed with pH Profile 1 and pH Profile 2, after 5h and 7h of the fermentation time and at its end (23.5h).

	Profile 1		**Profile 2**	
Time (h)	**OD_{600}**	**Compound A activity ($IU.g^{-1}$)**	**OD_{600}**	**Compound A activity ($IU.g^{-1}$)**
5	2.44	1780	2.34	1090
7	5.03	6460	5.38	5450
23	5.05	6840	3.88	3000

Observing the results of table 4.5, it appears that in profile 1 the cell growth and the compound A activity values showed no significant improvement, compared to the reference fermentation. Although the results still need to be confirmed, this may suggest that it would be a cheaper fermentation since after a certain amount of time no more base was needed, to obtain the same levels of compound A.

On the other hand, profile 2 showed no improvements on compound A production contrary to what was shown in literature. It may be possible that the design needs to be improved since 10 minutes may not be long enough for the pH to drop sufficiently.

However, these pH profile experiments were only tried once (fermentation profiles in Appendix 4) what means that they should be repeated more often to get more reliable conclusions.

4.4 Effects of varying the medium composition

The reference medium used so far is a simple medium that provides a good growth of this *L.lactis* strain and consequently, good compound A titres. However, the nitrogen source used, the Yeast extract Paste, is the component that has the bigger contribute to the medium costs. YEP is the common name for a form of processed yeast products made by extracting the cell contents (removing the cell walls), which are used as nutrients for bacterial culture media. [5], [12]

In order to try to reduce the costs, some experiments were made with the purpose of reducing the amount of YEP used in the reference fermentations. Therefore, a Complex medium (CM), suggested in literature, was used with the concentrations presented in table 4.5. The experiments were performed in 0.5L Multifors by Infors HT, maintaining the other reference fermentation conditions and following to the protocol presented in Appendix 1.

Table 4. 6 - Complex Medium composition, suggested by literature.

Complex Medium [1]	**Concentration ($g.L^{-1}$)**
Sucrose	30
Yeast extract	10
Peptone	10
KH_2PO_4	10
NaCl	2
$MgSO_4.7H_2O$	0,2

To better understand the role of YEP in the medium, another experiment was done with the same CM but without peptone. This last component is an enzymatic digest of animal protein whose nutritive value is largely dependent on the amino acid content that supplies essential nitrogen.[13] In Appendix 4 the fermentation profiles of these experiments are shown.

The biomass formation, sucrose and lactate concentration and compound A activity values comparing the two CM fermentations (with and without peptone) with the reference fermentation are presented in table 4.6.

Table 4. 7 - Biomass, sucrose, lactate and compound A activity results (end of fermentation time) from the anaerobic fermentations performed with the reference conditions, varying the medium composition: Reference medium; Complex medium; Complex Medium without peptone.

	Reference Medium	**Complex Medium**	**Complex Medium - no peptone**
Biomass (OD_{600})	3.76	2.49	2.48
Sucrose ($g.L^{-1}$)	0.63	2.48	5.1
Lactate ($g.L^{-1}$)	27	28.8	27.9
Compound A activity ($IU.g^{-1}$)	6042	6320	5590

Observing the previous results, it appears that peptone is not needed as a nitrogen source, since compound A production doesn't suffer significant changes. Moreover, although the biomass values are shown to be slightly lower, the reduction of YEP concentration may be feasible to maintain the same compound A activity.

Once again, due to the limited time, these experiments were performed only once. It would be interesting to continue this studies to confirm the results and other experiments should be made, reducing the amount of YEP in the reference medium so the results could be better compared.

Another interesting study concerning medium composition would be to further study the effect of $CaCl_2$. It was shown in previous studies that it helps decreasing the tailing effect and that it improves compound A production, in some strains. However, its is also shown that it affects negatively the meat processing, so the purpose with be to reduce $CaCl_2$ concentration in the medium, as much as possible.[14]

5. Conclusions and Recommendations

Several experiments were performed to test the effect of cultivation conditions on *Lactococcus lactis* for the production of compound A. Its antimicrobial activity plays an important role in food preservation and so it is of best interest to achieve the best conditions for the bacteriocin production.

All anaerobic fermentations performed appeared to confirm the literature statements that compound A is produced as a primary metabolite, since it is directly related to cell growth. The highest compound A activity results were almost always obtained at the end of the exponential phase and its production frequently stopped when the cells entered the stationary growth phase. In this sense, it can be concluded that the end of the exponential phase would be the best time to harvest the broth, in order to achieve maximum specific compound A productivity.

The effect of the carbon source concentration in anaerobic fermentations demonstrated that low sucrose concentrations (1%, 2%) result in lower compound A activity values, as expected, because of the fast depletion of the C-source. On the other hand, concentrations higher than 4% of sucrose appear not to increase the compound A activity, possibly due to substrate inhibition. According to the experiments performed, 3% of sucrose seems to be the most effective concentration of substrate, since it appears to deliver shorter fermentation times with a reduced tailing effect an with unchanged compound A activity and, consequently, fewer costs associated.

However, all of these results should be confirmed with more experimental data to obtain more reliable conclusions and it would be interesting to test a different range of sucrose concentrations (e.g. 1.5, 2, 2.5, 3, 3.5%) to define the optimal concentration for this type of fermentation.

Varying the pH in anaerobic fermentations showed that at pH 5 the cell growth is inhibited and that compound A production is lower, as expected. On the other hand, at pH 7, compound A activity results were also low, possibly demonstrating the attachment of the bacteriocin to the cells. However, some studies were made previously to this work and adsorption to biomass could not be reproduced, nor its relationship to pH. Therefore, this effect should be tested again, in order to better understand what happens when the pH is higher than 6 and its influence on compound A production.

In the end, no big difference was found between pH controlled anaerobic fermentations at pH 5.5 and 6, meaning that this range constitutes the adequate one for higher compound A production.

Besides fermentations controlled at a constant pH, fermentations with pH profiles are suggested in literature as a way of improving compound A activity, where the pH drops freely after some fermentation time (no more base is added in that time – possibly reduced costs) or where the pH

control is turned on and off to create a positive stress for the production of compound A. However, a more profound study is recommended in order to demonstrate the effects of these pH profiles.

The medium composition is one parameter of great importance on compound A production since it influences greatly the biomass formation and the purpose is to find the best recipe (supplying the carbon and nitrogen sources and all the nutrients necessary for cell growth) that is cost effective. In this sense, some experiments were done reducing the YEP concentration in the medium, since it's the compound with largest influence on the medium costs. Comparing the results of using a complex medium (CM) with half of the YEP concentration and the same medium without peptone (the other nitrogen source besides YEP), it appears that compound A activity values were very similar. This fact may indicate that no other nitrogen source is needed, besides YEP, and that the reduction of its concentration may be feasible, with similar levels of the bacteriocin.

As mentioned above, these conclusions were made regarding the complex medium. It is, then, recommended that the same study is performed with the reference medium used in the other anaerobic fermentations, to confirm if the reduction of YEP is feasible.

Another recommendation, concerning the medium composition, would be to perform further investigation on the influence of reducing the amount of $CaCl_2$ added. Previous studies showed the importance of adding this component, since it reduces the tailing effect during fermentation and it appears to slightly improve compound A production, in some *L. lactis* strains. However, it was also shown to affect negatively the meat processing and therefore no/or minimal amount of $CaCl_2$ is desired in the compound A end product.

Several other parameters may also influence the biomass formation and could not be performed in this study, like the type of base added to control the pH (or the addition of a mix of different bases) and the type of substrate (e.g. glucose), for example. It would also be interesting to test its effect on compound A production in order to obtain the best, cost effective, recipe.

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Appendices (Confidential)