Norwegian University
of Life Sciences

Master's Thesis 2021 30 ECTS
Faculty of Chemistry, Biotechnology and Food Science

Growth and metabolism of sporeformers in cheese

Antonella Cingano
Food Science

Acknowledgments

This master thesis was written during the Covid-19 pandemic and has been affected by the reduced accessibility to the laboratories. Nevertheless, the entire team of the Dairy Technology and Food Quality department have been going the extra mile to provide me with the best conditions possible to accomplish my task.

I would like to thank my relators, professor Siv Borghild Skeie and Professor Davide Porcellato, for their guidance and all the support they have showed me even in the most difficult times. Professor Skeie, not only in the process of this thesis but through the years, has been showing a rare combination of formidable competence, experience and great pedagogic skills. Davide, I thank you for the great availability you have showed all the way. Your enthusiasm and energy have made working with you a great pleasure.
This master thesis could not have been accomplished without Senior Engineer Ahmed Abdelghani, who has been guiding my work in the lab with competence, kindness and a patience of biblical proportions. I owe him much and I am deeply thankful.
A must felt thanks goes to Senior Engineer Kari Olsen, which was of such big help in the performance of chemical analysis.
A big thanks goes also to Engineer May Helene Aalberg for the practical help and for the good vibes she spreads.
I thank all those of the lab team that in different ways have taken part in this work.

At last, I would like to thank my children Edda and Nilo for all the support, the inspiration and the joy.

Antonella Cingano, Oslo, October 2021.

Abstract

Spore formers represents a great challenge for the dairy industry, as the spores produced by these bacteria can withstand heat treatment and may be a cause of spoilage of the product.

Members of the *Bacillus cereus* group and of the *Clostridium sensu strictu* cluster may, with their metabolic and enzymatic productions, be detrimental for the quality of cheese and its shelf life, thus causing the dairy industry important economic losses.

This master thesis aimed to define growth and metabolic pathways of selected isolates in cheese, focusing on their casein degradation ability and the utilization of amino acids, organic acids and carbohydrates as energy sources.

Metabolic differences between isolates of *Bacillus cereus, Clostridium sporogenes and C. tyrobutyricum* were defined in UHT milk and in a cheese model.

The results showed that *B. cereus* and *C. sporogenes* produce proteolytic enzymes and use amino acids as energy source,in combination with organic acids and carbohydrates.
C. *tyrobutyricum*, on the other end, utilizes only organic acids and carbohydrates and shows low proteolytic activity.

1. Introduction.

1.1. Milk composition

Milk represents a good substrate for many microorganisms. In addition to high water activity and neutral pH, its content of proteins, fat, lactose, minerals and NPN allows several microbiological metabolisms (Walstra, 2014).

Milk is an aqueous solution of lactose, organic and inorganic acids, vitamins and small molecules, which comprises a system of dispersed protein aggregates and emulsified lipids.
Typically, bovine milk contains 3.7% fat, 3.4% protein and 4.8 % lactose (Fox et al., 1998), although the content of components and chemical characteristics show great variation depending on several factors, such as genetic features, health and age of the animal, nutritional status, breed, lactation stage, season, type of feed and intervals between milking sessions.
The main milk carbohydrate, lactose, is a disaccharide made of one molecule of glucose and one molecule of galactose. Other sugars such as glucose, fructose, gluco- and galactoseamine and N-acetyl neuraminic acid are present in form of oligosaccharides in trace amounts.
The protein fraction is made for the 20 % of small soluble proteins referred to as whey proteins and for the 80% of caseins, of which 95% are present as large colloidal complexes called micelles.
An overview of the content of the milk protein fractions is given in Table 1.

Table 1. Major proteins in milk. Fractions content is given in grams/liter milk and as percentage of total protein content (Walstra & Jenness, 1984).

Major milk proteins	Grams/Liter	% of total protein
Total protein	33	100
Total caseins	26	79.5
α_{s1}	10	30.6
α_{s2}	2.6	8.0
β	9.3	28.4
K	3.3	10.1
Total whey proteins	6.3	19.3
α-lactalbumin	1.2	3.7
β-lactoglobulin	3.2	9.8
BSA	0.4	1.2
Immunoglobulins	0.7	2.1
Proteose peptone	0.8	2.4

The major whey proteins are β-lactoglobulin (β-lg), α-lactalbumin (α-la), blood serum albumin (BSA) and immunoglobulins (Ig). Other whey proteins, such as lactoferrin, serotransferrin and enzymes, are present in trace amounts.

Caseins are divided in α_{s1}, α_{s2}, β and κ fractions.

The proteose peptone fraction results from proteolytic activity of plasmin on β casein and is found outside the micelles.

In addition to whey proteins and caseins a nonprotein nitrogen fraction (NPN), composed of urea, peptides and free amino acids, is present in trace amounts.

Milk lipids present an extremely varied fatty acid composition and exist mainly in the form of fat globules, with a core of triglycerides surrounded by a fat globule membrane rich in phospholipids (Walstra, 2014).

1.2. Cheese.

Nowadays the worldwide production of cheese counts thousands of varieties, which differ in manufacturing method, degree of ripening, nutritional and microbial content, and sensorial and organoleptic properties. Among all varieties, the semi hard types Cheddar, Dutch and Swiss and the *pasta filata* cheeses dominate the market (McSweeney et al., 2017).

Cheese is, in general terms, the result of casein aggregation and drainage of whey.

Caseins aggregation is caused by loss of the negative charge on the surface of the micelles. This can be achieved either by lowering the milk pH down to the isoelectric point of casein by means of acid addition, or by enzymatic hydrolysis of k casein. Enzymatic or rennet coagulation is used in the manufacture of the majority of cheese types and account for the 75% of cheese production. Acid coagulation is used in the production of some fresh cheeses, such as cottage cheese, quarg and labaneh and, in combination with heat treatment, of ricotta, mascarpone and queso blanco (McSweeney et al., 2017).

The general steps of the cheese making process for rennet-coagulated cheeses are shown in Figure 1.

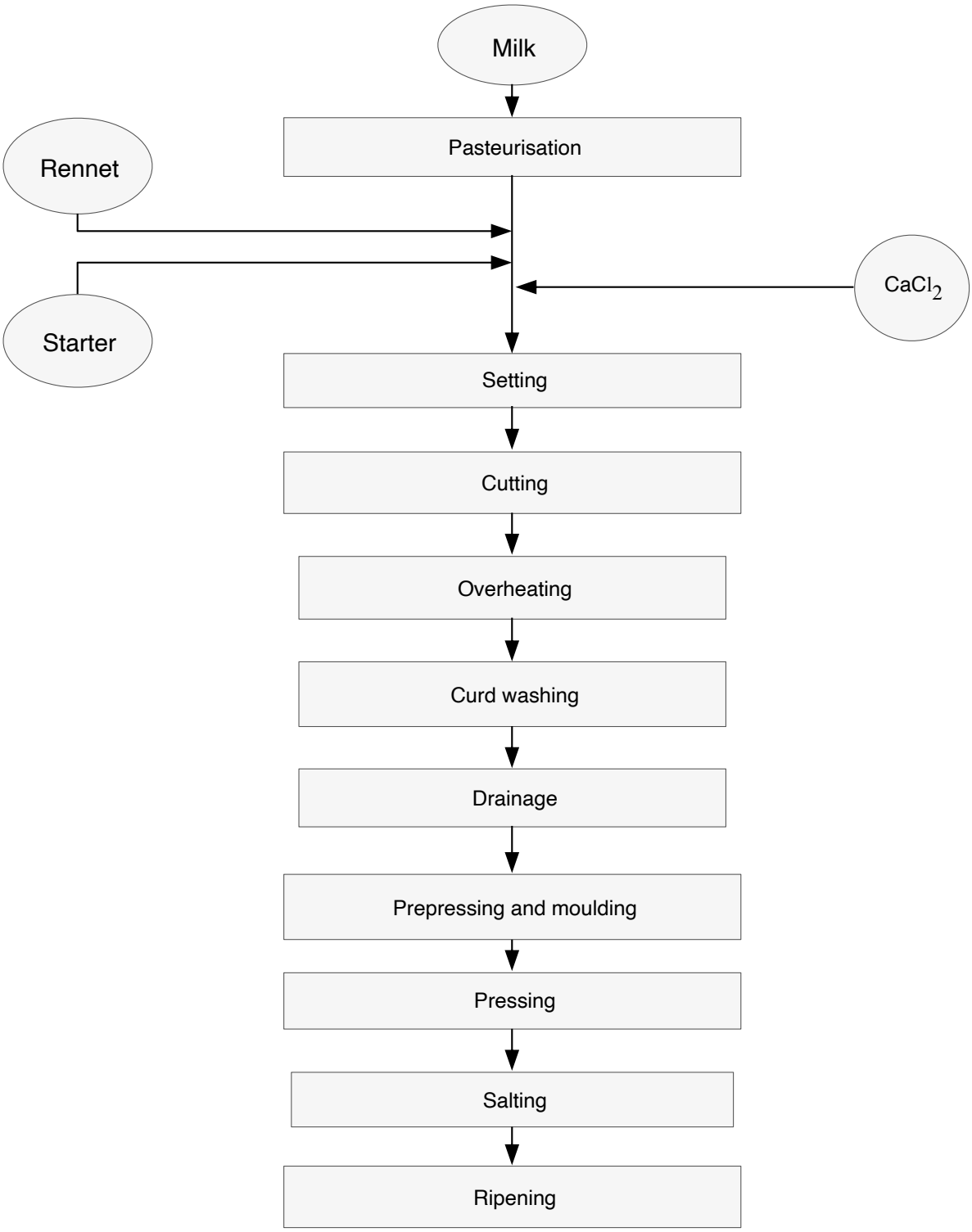


Figure 1. Steps in the cheese making process (Ferragut & Trujillo, 2008).

Rennet coagulation may be divided in several phases. During the primary phase, or enzymatic phase, the glycomacropeptide fraction of K-casein is removed by the enzyme from the micelles' surface, thus compromising the stability of the colloidal system. At temperatures above 20ºC, the micelles coagulate, aided in this by binding of calcium ions, and gradually

form a network. As the protein network grows and entraps fat globules and the milk serum, a gel is formed (secondary phase or curdling). Curdling is affected by rennet amount (usually 20-40 ml/100l cheese milk), content of calcium ions in the milk, temperature and pH. The renneting time, the amount of time between rennet addition and milk flocculation, is usually between 30 and 40 minutes. During this time the milk should be left undisturbed.
Rennet addition to the milk can be either at the same time or next the addition of the starters.

Starter cultures of lactic acid bacteria (LAB) are used to lower the pH, as they produce organic acids from degradation of lactose. In addition, their enzymatic and metabolic productions will confer the cheese the desired consistency and organoleptic properties. Starter cultures used in cheese making are either mesophilic or thermophilic. Mesophilic starters are combinations of strains belonging to *Lactococcus lactis lactis*, *L. lactis cremoris, L. lactis lactis diacetylactis* and *Leuconostoc mesentoroides cremoris.* Thermophilic cultures, use in the production of hard cheeses such as Emmentaler and Grana Padano, belong to the species *Streptococcus thermophilus*, *Lactobacillus helveticus* and *L. bulgaricus.*

The third phase of rennet coagulation consists of syneresis, or release of whey from the curd. This is achieved by cutting and stirring of the gel and by means of the overheating step.
Cutting is made when the gel achieves the desired firmness, which is usually evaluated by the cheese makers on the basis of subjective experience. Cutting of the curd can be fine or coarse, where fine cutting results in increased syneresis and it is used in the manufacture of hard cheeses.
After cutting, whey release from the curd is improved by continuous mechanical stirring, whey acidification and increased temperature, where temperature plays a major role in regulating of the cheese final moisture content.
Washing of the curd with water may be performed in order to adjust cheese and whey acidity.
Whey drainage can happen in several steps. In Norway whey from the first drainage is used in the manufacture of brown whey cheese.

Molding and pressing procedures vary in accordance with the desired cheese texture. In the manufacture of cheese with eyes, the cheese is collected and pressed under whey, whereas cheese with open texture undergoes molding after whey drainage. Pressing gives form and cohesiveness to the cheese and promotes rind formation.

Salting regulates the activity of the starter and the fermentation rate. Salt can be added directly in vat, in the cheese curd, in the form of brine after pressing or by means of dry salting.
Most rennet coagulated cheeses undergo ripening, which duration varies between two weeks and over two years according to the cheese type (Walstra, 2014), (Fox et al., 1998).

1.3. Cheese ripening and proteolysis.

During ripening chemical, biochemical and microbiological changes take place in the curd, which shape texture, flavor and aroma of the final product. Some of these changes are common to all cheese varieties, while others are specific to the cheese type. These changes are mainly to ascribe to four reactions: lipolysis, proteolysis, glycolysis and degradation of citrate.
Proteolysis of the casein matrix and its degradation to peptides and amino acids alter the protein water binding property and cause softening of the cheese texture. In addition, the short peptides and amino acids produced serve as substrate for several microbiologic metabolisms whose final products have great impact on cheese flavor. The primary sources of proteinases and peptidases in cheese are residual rennet, proteinases indigenous to the milk, LAB, secondary starters, non-starter lactic acid bacteria (NSLAB) and, in the case of smear cheeses, surface bacterial flora. In general, the casein matrix is mainly hydrolyzed in peptides by indigenous proteinases and residual rennet, the latter being mainly active on α_{s1} casein, while LAB, NSLAB and the secondary microflora are responsible for degradation of peptides to amino acids (McSweeney, 2004) and (Fox et al., 1998).

1.4. Sporeformers contamination of the dairy production chain.

Thanks to the resiliency of their spores, spore formers are ubiquitous in nature. Species belonging to the *Bacillus cereus* group have adapted to a wide variety of ecological habitats: endemic in soil, they have been isolated in plants and in the intestinal tract of different animal species (Stenfors Arnesen et al., 2008). Similarly, *Clostridium* spp. are to be found in very diversified environments, from water to soil to the digestive tract of mammals (Brändle et al., 2016).
Sporeformers contamination interests a large variety of products and ingredients, both of vegetable and of animal origin. Contamination of the food production line appears to have its main origin in soil and in plant material, from which vegetative cells and spores spread further to the production areas and the equipment. Cross contamination between products is also a common problem in the food industry (Stenfors Arnesen et al., 2008).

The Bacillus cereus group and several clostridial species have been identified by gene sequencing as members of the core microbiota of raw milk, although their proportion show high variability (Kable et al., 2016).
Contamination of spore formers in raw milk takes place already in the farm through the pathway illustrated in Figure 1.

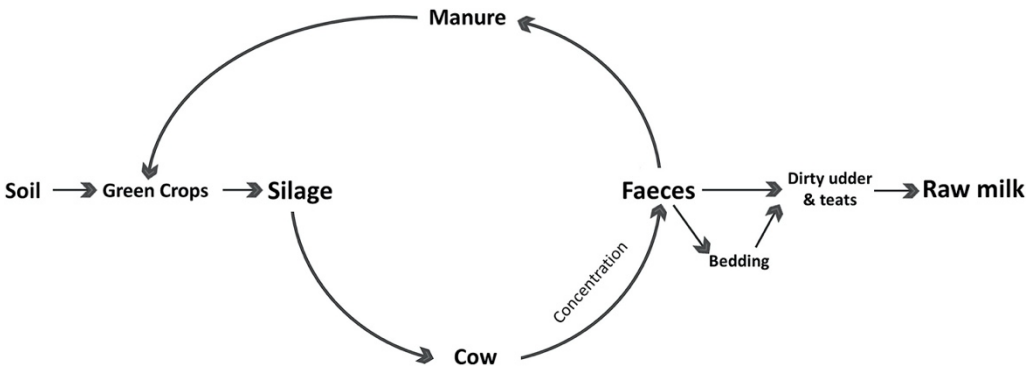


Figure 2. Pathway of spore formers contamination from soil to raw milk (Brändle et al., 2016).

From soil and green crops, spore formers enter the digestive system of the cow with silage. Eventually, faeces vehiculate contamination further to the bedding material, the feed, and finally the udder, as well as to the crops used for manure, thus restarting the contamination cycle (Brändle et al., 2016).

A key role in the contamination pathway of raw milk is played by silage fermentation which, if incomplete, may cause major growth of sporeformers. Measures aimed to reduce silage-connected risks have been taken to various extends, from improvements of the ensiling process as such and of the monitoring systems to, in the case of some alpine regions, the prohibition of the use of silage as feed.

Despite the attention placed into good farming practices and hygiene improvements under milking, transport and storage, spore formers contamination of raw milk is still an unsolved problem (Gómez-Torres et al., 2015), (Brändle et al., 2016), (Brändle et al., 2018).
From raw milk, thanks to their thermoresistant spores, spore formers have easy access to the downstream of the dairy processing, causing spoilage in different productions.

Another big concern for the dairy industry is represented by the ability of B. cereus to form biofilms, which can themselves become with time spore reservoirs and are particularly challenging to remove from the processing equipment, especially pipes .
In addition, due to their high hydrophobicity, spores adhere to surfaces better than vegetative cells and their removal requires specific sanitation (Huang et al., 2020) , (Stenfors Arnesen et al., 2008).

1.5. Cheese milk quality requirements and methods in use to reduce spore number in the cheese.

Raw milk, whether destinated to become milk for consumption or used for other dairy processes, must not contain traces of chemicals, detergent and antibiotics, must be of high hygienic quality and present low cell number. As mentioned above, feed quality and hygienic standards along the raw milk chain are determinant in the avoidance of microbial contamination. At reception in the dairy implant raw milk is subject to chemical and microbiologic controls. The prescribed limit for number of bacteria in raw milk is 100 000 colony forming unit /ml. If higher cell number or presence of antibiotics are detected, the milk will be destroyed (Ruegg & Reinemann, 2002) and (Walstra, 2014).
Cell number is given by leukocytes and epithelium cells. In cheese production a high cell number gives increased dissolved caseins in the serum phase, increased renneting time, increased fat loss in the whey, reduced cheese yield and increased water content in the cheese. Milk destignated to cheese production has also to fulfill other specific requirements. It should have high protein content and have suitable genetic variants, as genetic variance affects casein content, caseins ratio, k casein glycosylation and salt balance (Walstra, 2014) .
Milk pasteurization, usually performed applying a temperature of 72º for 15 seconds, is meant to destroy undesired vegetative cells, thus ensuring the milk microbiological safety and longer shelf life. As pasteurization doesn't provide spore inactivation, additional processing can be applied to cheese milk in order to limit spore content (Walstra, 2014) .
Bactofugation is a super centrifugation at 9-10000 g that separates spores from milk on the basis of their molecular weight. Bactofugation can be performed in several steps, consenting to remove up to 99% of the spores. Bactofugation, however, does not provide full spore elimination.
Cheese milk can alternatively undergo microfiltration, a type of membrane separation which effectively separates the milk from bacteria, spores and dead cells. The downside of

microfiltration is that it can be performed only on skimmed milk, as fat can clough the filtering membrane.
Germination of spore formers can also be mitigated by the addition of chemical and biochemical compounds, such as nitrate, lysozyme and nisin.
Nitrate addition is usually performed after the first whey drainage. Nitrate prevents growth of coli formers and clostridia, although it may have negative effects on LAB. In addition, nitrate addition may cause formation in the cheese of nitrosamine, which are cancerogenic.
Lysozyme and nisin attack the cell wall lysis in Gram positive bacteria, but their efficacy on spore formers is not completely satisfactory (Gómez-Torres et al., 2015) and (Brändle et al., 2016).

1.6. Sporeformers in milk and cheese.

Under cell division vegetative cells produce proteolytic, lipolytic and phospholipolytic enzymes which are able to survive at low and high temperatures. These enzymes are a great concern for the dairy industry as they impact the technological and sensorial attributes of the products, being often the cause of off flavors and reduced shelf life (Walstra, 2014).

Bacillus cereus proteases are mainly responsible for sweet curdling of milk, gelation of UHT milk and for reduced solubility of milk powders. Together with lipases, these are also accountable for bitterness in these products (Mehta et al., 2019). The lack of literature about Bacillus cereus group spoilage in cheese may suggest that the main concern related to cheese contamination by these bacteria is that of microbiological safety.
Clostridia do not find in milk suitable conditions for growth (Podrzaj et al., 2020). Inversely, in cheese, nutrients availability, favorable temperature conditions and an anaerobic environment lead to spore germination and outgrow of vegetative cells (Walstra, 2014).
Clostridia are the major cheese spoilage agents, and their impact on cheese production - and cheese producers - is of vast proportions. Some *Clostridium* spp. can in fact produce H_2 and CO_2 in high concentration by means of lactate fermentation, which may results in slits, defects in eye formation and in abnormal blowing of the cheese forms, the so called late blowing defect (LDB). In addition, the acids produced during butyric fermentation has a detrimental effect on cheese flavour (D'Incecco et al., 2018). An example of LBD in Gouda cheese is given in figure 3.

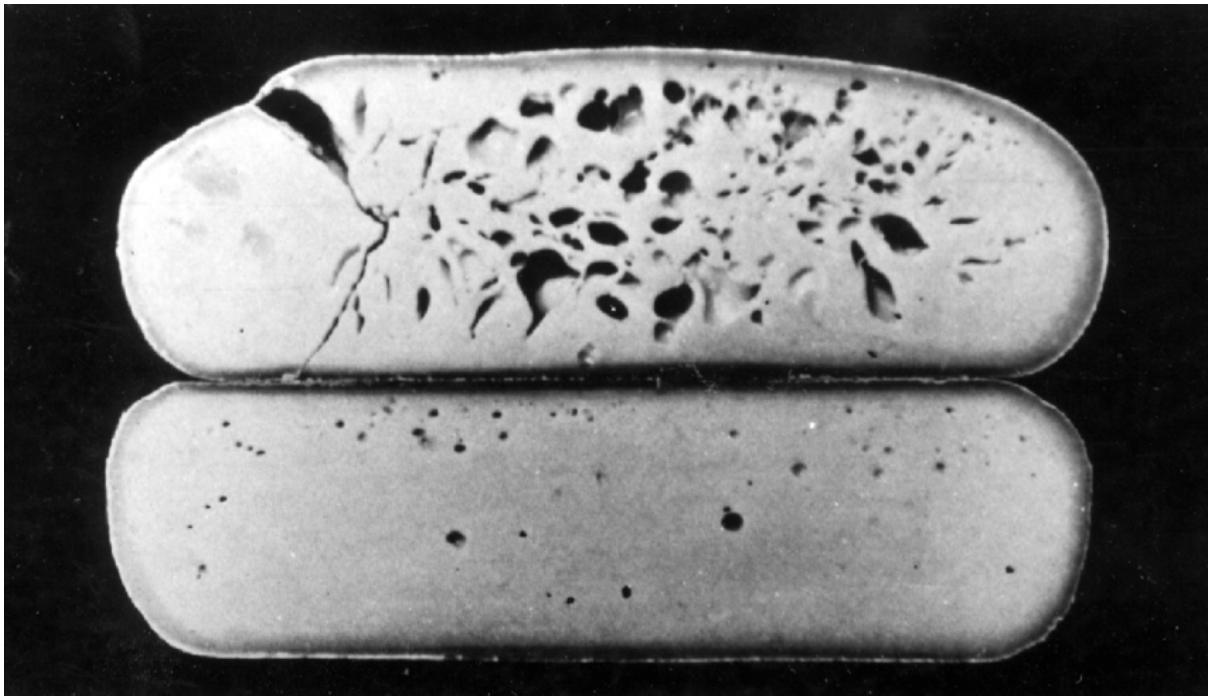


Figure 3. Late blowing defect (LBD) in cheese. Cheese of normal quality (bottom) and cheese affected by LBD (Pahlow et al., 2003)

Butyric fermentation in cheese is influenced by spore amount, pH, dry matter, salt content and metabolic interactions with other bacteria, as well as by the scalding temperature of the curd and that of the ripening room (Walstra, 2014) and (Fox et al., 1998).

Traditionally, *C. tyrobutyricum* has been considered the main spoiling agent of cow milk cheese with regard to late blowing. This species owes its name to the fact that it was the first isolated in blown cheese, as *tyron* means cheese in ancient Greek (Brändle et al., 2016). Isolation of other Clostridia in spoiled cheese may suggest an interactions with strains of *C. beijerinckii*, *C. butyricum* and *C. sporogenes* (Brändle et al., 2018).

However, a study conducted on species effect on late blowing indicated *C. tyrobutyricum* as the most aggressive spoilage agent with the greatest production of butyric acid, CO_2 and H_2 (Gómez-Torres et al., 2015).

1.7. The *Clostridia sensu strictu* cluster.

Part of the phylum Firmicutes, *Clostridium* spp. represent a vast and heterogenous genus which consists of over 200 species, nowadays classified in subgroups in accordance with 16S rRNA sequencing. *Clostridia sensu strictu* are a cluster of Gram positive, mostly strict anaerobe rods

of which *C. botulinum* representing the cornerstone species. Members of the cluster have been detected in dairy products already at the beginning on the 20th century. Within the group, *C. perfringes* and *C. botulinum* are major causes of food borne illnesses, the first being responsible of common gastrointestinal infection, the second being the etiological agent of the most lethal botulism (Doyle et al., 2015).

However, the *Clostridium* spp. which, causing late blow defect, pose a severe challenge to the dairy industry, are those able of butyric fermentation - the butyric acid bacteria (BAB). Although not regarded as BAB, *C. sporogenes* has been also found responsible of gas production in cheese (Doyle et al., 2015)

Clostridia *sensu strictu* show great metabolic variety. Some species are able of carbohydrate metabolism, whereas others utilize organic acids and amino acids as carbon source. Substrate preference and metabolic products ratio are both species and strain related (Dash et al., 2016). The central metabolism for saccharolytic clostridia is shown in Figure 4.

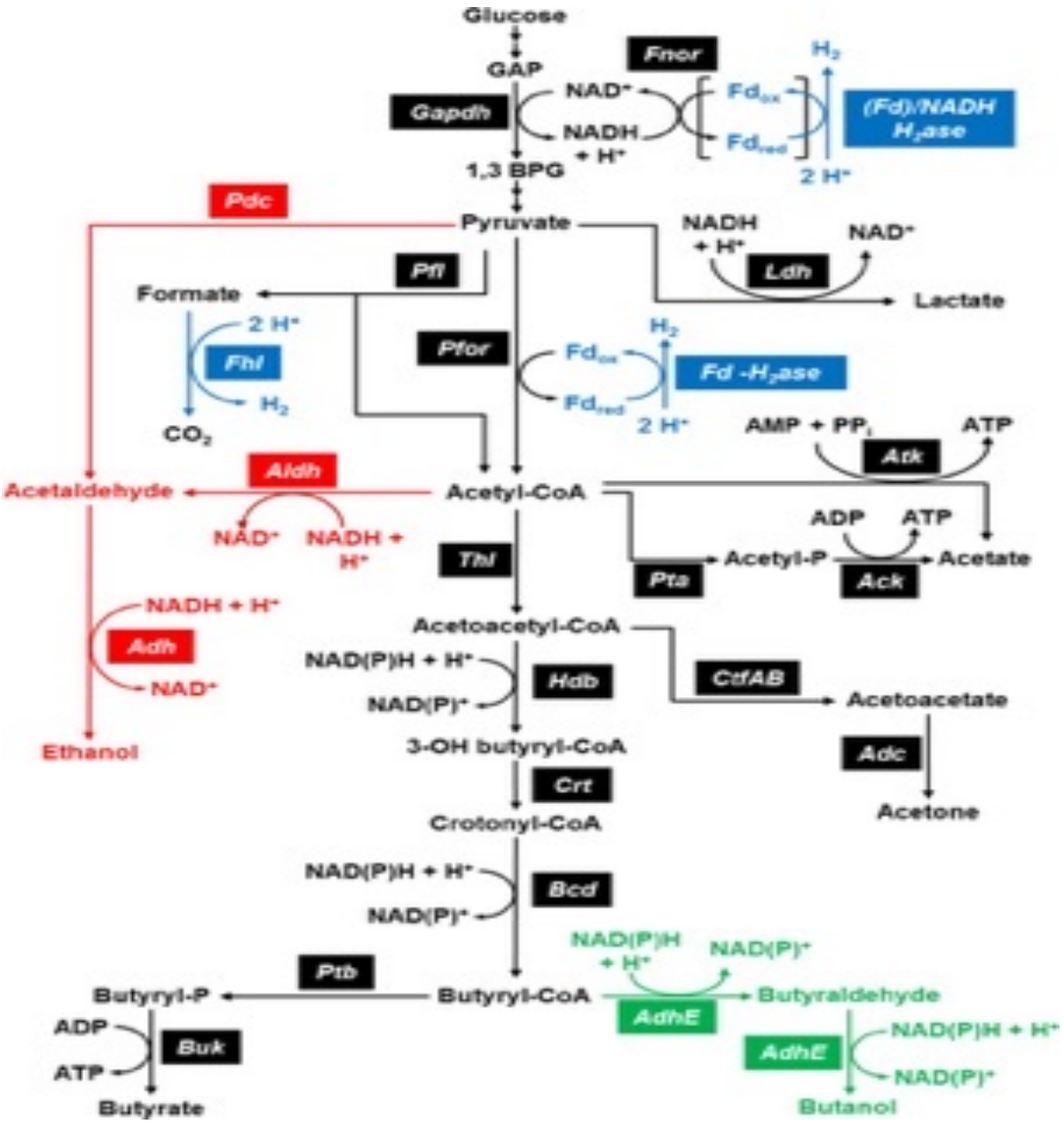


Figure 4. Overview of the central metabolism of Clostridia ssp. The pathways for the different end-products are given as following: in black that for butyrate, acetate and acetone, in red that for ethanol, in green that for butanol and in blue that for H2 (Mazzoli, 2012).

Glucose is converted into pyruvate with release of H2. Pyruvate is then converted to lactate, to ethanol via acetaldehyde, and to formate and acetyl-CoA with release of H2 and CO_2. Acetyl-CoA is converted in acetaldehyde, in aceto-acetyl-CoA and in acetate. Aceto-acetyl-CoA is converted to acetone via acetoacetate and to butyryl-CoA via 3-OH butyryl-CoA and crotonyl-CoA. Butyryl-CoA is converted into butanol via butyraldehyde and into butyrate via butyryl-

P. ATP is generated by conversion of acetyl-CoA into acetate and of butyryl-P in butyrate. (Mazzoli, 2012).

In the absence of glucose, some species are able to perform lactate metabolism, converting lactate into pyruvate. From pyruvate, metabolic pathways are similar to those occurring in glucose metabolism, as shown in Figure 5.

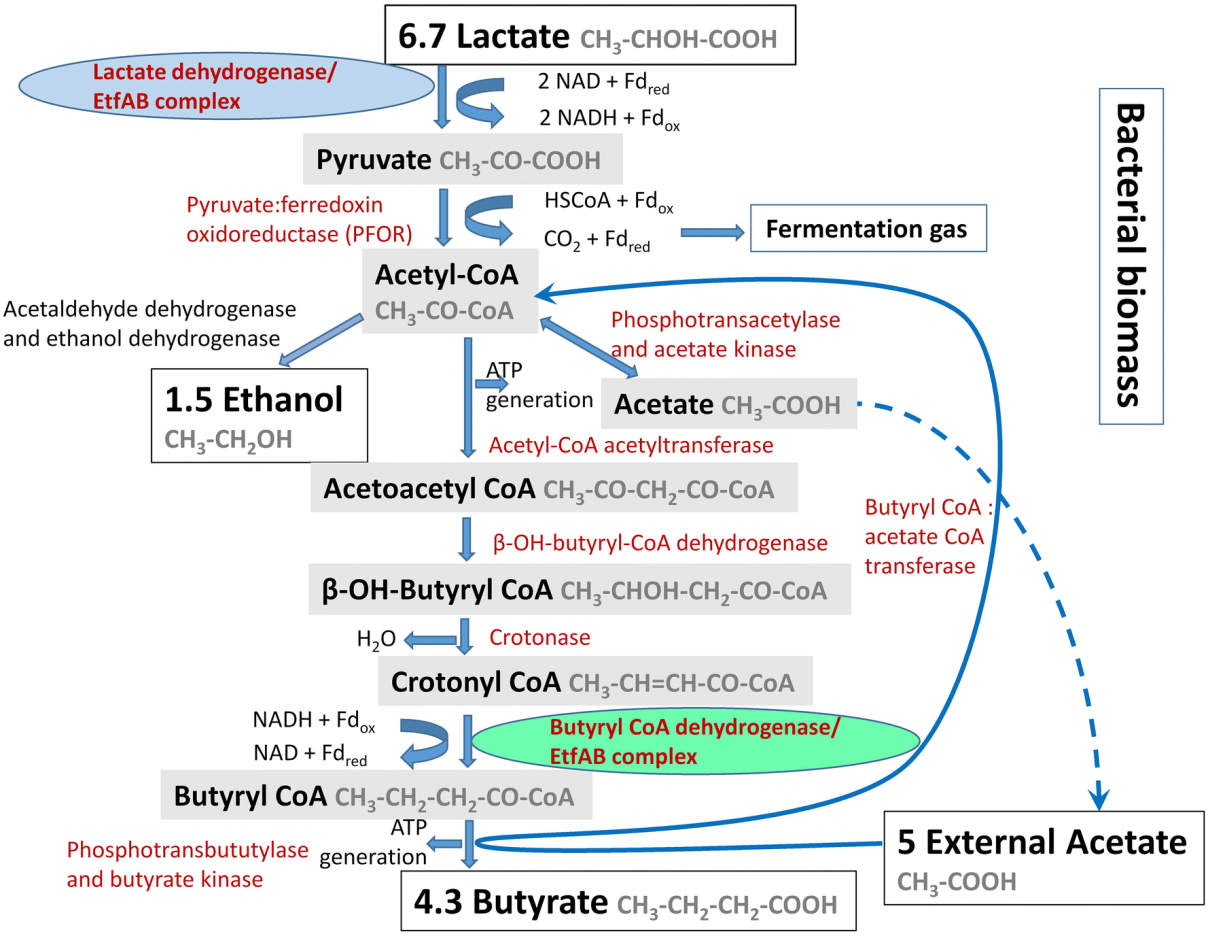


Figure 5. Overview of lactate pathway in Firmicutes (Detman et al., 2019).

Lactate is converted into pyruvate and further into acetyl- CoA with release of CO2. From acetyl-CoA ethanol, acetate and aceto-acetyl-CoA are produced. Aceto-acetyl-CoA is further converted into the intermediate products β-OH- butyryl CoA, crotonyl-CoA and butyryl-CoA and finally into butyrate.

Acetate is either converted into butyrate or returns in the pathway's downstream as acetyl-CoA. ATP is generated under conversion of acetyl-CoA into aceto-acetyl-CoA and of acetate and butyryl CoA into butyrate (Detman et al., 2019).

Even when glucose is present, some clostridial species as for instance *C.sporongens*, utilize amino acids as the preferred carbon source (Storari et al., 2016). This type of fermentation, known as Strickland fermentation, is a 2 steps redox reaction which involves specific pairs of amino acids, where the one acts as an electron donor and oxides the other. An example of Strickland reaction is given in Figure 6.

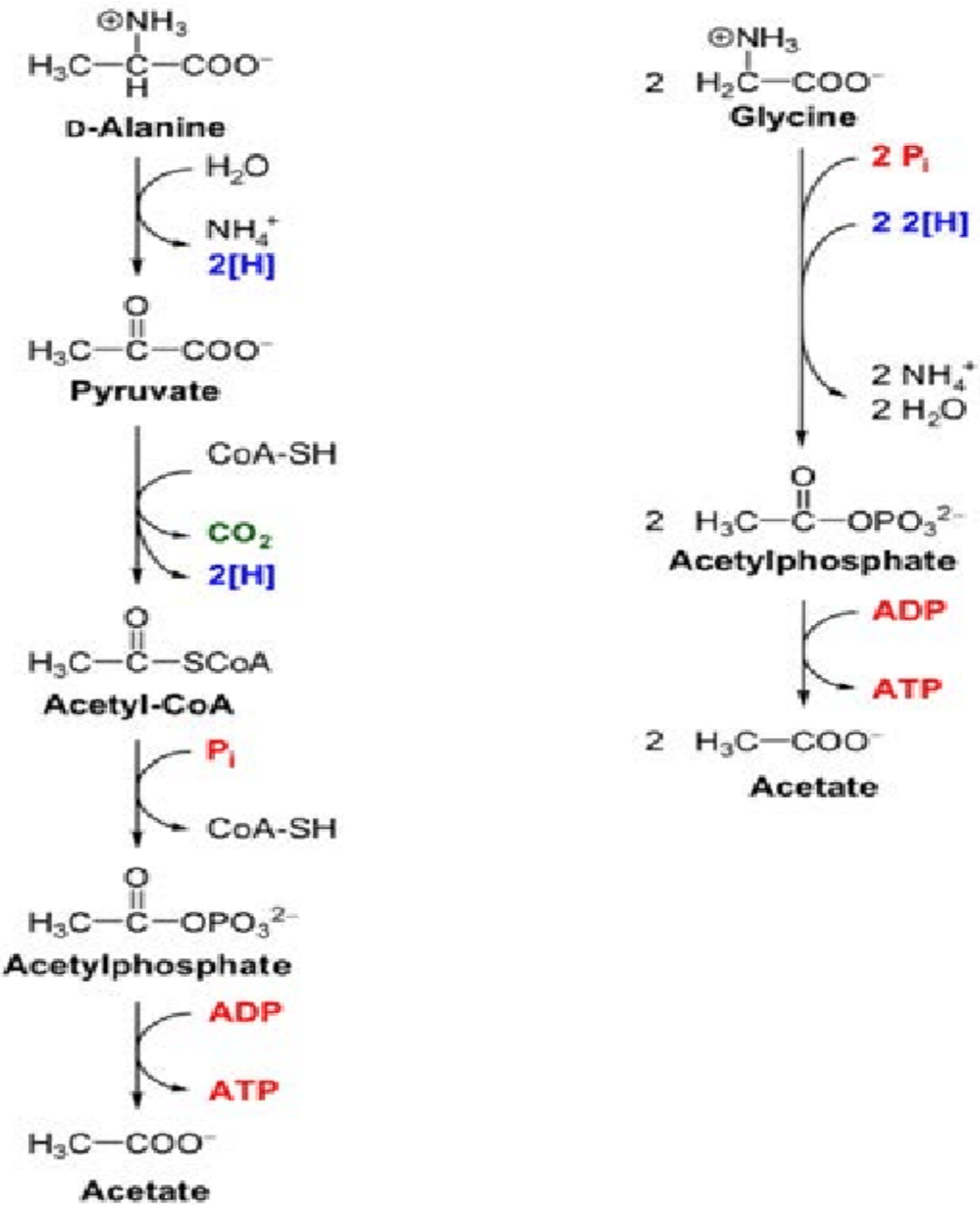


Figure 6. Strickland reaction between D-alanine and glycine. On the right, oxidative deamination of d-alanine and on the left reductive deamination of glycine (Piveteau, 2017).

Oxidative deamination gives pyruvate as an intermediate product, which is converted into acetyl phosphate by binding of inorganic phosphate. In reductive deamination, deamination and phosphorylation happen in one step. Acetyl phosphate is then used as substrate for ATP synthesis, while converted into acetate (Piveteau, 2017).

1.8. The *Bacillus cereus* group: taxonomy and ecology.

"*Bacillus cereus* group" is an umbrella term which currently defines 8 higly genetically conservative species within the genus *Bacillus*: *B. cereus sensu stricto, B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, *B. anthracis*, *B. weihenstephanensis*, *B. citotoxicus* and *B. toyonensis*. The genetic affinity of these species has been shown by 16s rRNA gene sequencing to be even above 99%, the margin of difference being represented by genes encoded in plasmids (Porcellato et al., 2018), (Stenfors Arnesen et al., 2008).

Chromosomal similarity, together with the high mobility of plasmids as genetic elements and the horizontal gene transfer occurring on strain level, has made taxonomic identification of the single species challenging. In addition, great incongruency exists between phylogenetic studies and the group's ecological heterogenicity, which keeps the debate on whether considering the Bacillus cereus group as one single species still open. Plasmid-encoded genes are in fact responsible for great behavioral variation within the group, especially in terms of pathogenicity and of temperature resistance (Stenfors Arnesen et al., 2008).

B. antracis, *B. cereus sensu strictu* and *B. thuringensis* have high pathogenic potentials. *B. thuringensi*s 's endotoxins are lethal for larvae of various insects and this species is largely used in the production of biopesticides. Traditionally, *B. anthracis* is identified as the etiological agent of the lethal disease anthrax, although also some *B. cereus sensu strictu* strains have been shown to cause anthrax-like infections (Ehling-Schulz et al., 2019). Moreover, *B. cereus sensu strictu* is one of the major responsible of food borne diseases worldwide, as this species can cause both an emetic intoxication and a diarrheal infection (Stenfors Arnesen et al., 2008).

As for temperature resistance, although the majority of species within the group have mesophilic traits, *B. weihenstephanensis* and some strains belonging to *B. cereus sensu strictu* and *B. thuringeiensis* can grow at temperatures below 7 °C, while *B. citotoxicus* is thermotolerant (Stenfors Arnesen et al., 2008). The group shows growth in a broad temperatue ranging from 4°C to 50°C, being for this reason an issue for many food productions (Guinebretière et al., 2008).

1.9. *Bacillus cereus* metabolism.

Members of the *Bacillus cereus* group can grow both in aerobic and anaerobic conditions thanks to their metabolic flexibility (Duport et al., 2016). An illustration of the central metabolism of B. cereus is given in Figure 7.

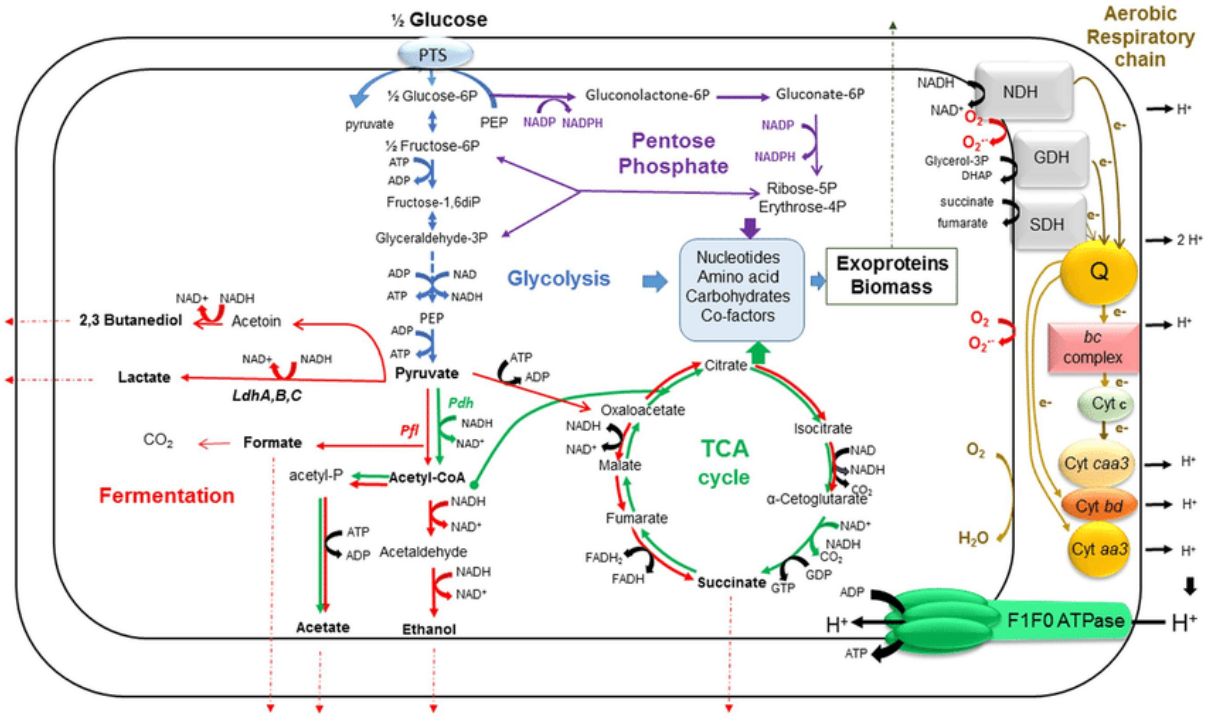


Figure 7. Metabolic flexibility of B. cereus. Glycolysis is indicated in blue, the pentose phosphate pathway in purple, the TCA cycle in green, the fermentative metabolism in red and the aerobic respiratory chain in brown (Duport et al., 2016).

In both anærobic and aerobic conditions, *B. cereus* ssp. catabolizes glucose primarily through glycolysis and secondarily through the pentose phosphate pathway (PPP). For each molecule of glucose-6 -phosphate glycolysis produces 2 molecules of pyruvate and 2 ATP, providing in addition a wide range of intermediates for biosynthesis. Ribose-5-phosphate and erythrose-4-phosphate from the PPP are essential precursors for biosynthesis of nucleotides, aromatic amino acids and histidines. PPP also fuels, among other reactions, biosynthesis of the detoxifying and antioxidant defense systems of the cell.

In aerobic conditions pyruvate is made into acetyl-CoA which enters the TCA cycle. The TCA intermediates oxaloacetate, α-ketoglutarate and succinyl-CoA are used in amino acids and phorphorines synthesis. The aerobic respiratory chain is a major provider of ATP, utilizing

NADH and FADH from glycolysis and TCA. The respiratory chain gives also origin to reactive oxygen species (ROS), which are highly oxidant.
When oxygen is reduced below the critic level for the aerobic respiratory chain to take place, glycolysis is upregulated in order to compensate the drop in ATP production. The activation of upregulating enzymes will induce the fermentative pathway.
In anaerobiosis, pyruvate undergoes the pyruvate-to-lactate pathway and is converted into 2,3 butandiol and lactate. In addition, pyruvate is converted into formate and, with acetyl CoA as intermediate, into acetate and ethanol. The main product of the fermentative metabolism is lactate. Ethanol and 2,3 butandiol are minor metabolic products at neutral pH, while in acidic conditions, production of butandiol will increase at the expenses of that of acetate and ethanol. Under anaerobic conditions, *B. cereus* spp. produces ATP by means of nitrate ammonification, in which nitrate from the environment is reduced first to nitrite and then to ammonia. However, production on ATP under anoxia is drastically lower than that observed in aerobic conditions. Ammonia is also produced under acid stress condition, as it is used as internal buffer. In this case ammonia can be produced by hydrolyzation of urea by the urease system and the catabolization of arginine by the arginine deiminase pathway. As the arginine deiminase pathway is also source of ATP, bacteria provided of this pathway can eventually rely on the only on arginine for growth (Duport et al., 2016).

1.10. Spore formers, a challenge for the dairy industry.

Spore forming bacteria represent a great challenge for the dairy production chain worldwide. Beyond the pathogenicity of some species, which may be the cause of foodborne illnesses, these bacteria are able to compromise the shelf life of the products and are responsible for major quality defects, especially in cheese. As a consequence, they may cause considerable economic losses for the industry (Doyle et al., 2015).

The main concern related to spore formers resides in their ability to produce spores which are highly resilient to nutrient limitations and environmental stresses such as radiation, osmotic pressure and exposure to chemicals. Moreover, spores are not affected by the temperature treatments commonly performed in dairy processing, such as thermization and pasteurization. At favorable growth conditions and with availability of nutrients, spores eventually germinate and convert into vegetative cells of which metabolic and enzymatic activities are detrimental for product quality (Doyle et al., 2015).

Among spore forming bacteria, species belonging to the *Bacillus cereus group* and to the *Clostridia sensu strictu* cluster occur in dairy products with the highest frequency and are recognized as major spoilage agents (Doyle et al., 2015)

1.11. Aim of the thesis.

The CLOBIO project aims to increase knowledge on composition and survival of spore formers bacteria in Norwegian milk and milk products. The project is part of the food microbiology and food microbiota studies conducted by the Norwegian University for Life Science (NMBU) in collaboration with TINE.

Within the frame of the CLOBIO project, the present thesis aims to investigate growth and metabolic pathways of spore formers in cheese.
Metabolic differences among selected isolates belonging to the *Clostridium sensu strictu* cluster and to *Bacillus cereus group* have been defined in terms of proteolytic activity in milk and of the production of organic and amino acids in a cheese model. The results achieved, placed in relation to quality problems commonly found in industrial cheese production, will hopefully provide solutions to the challenges these bacteria represent for the dairy industry in terms of hygienic safety and economic loss.

Efforts have already been made in order to isolate and identify by means of 16S rRNA sequencing spore formers species downstream the production lines for cheese and milk powders, in the attempt to determine how these bacteria can survive different processes and in which measure they may impact product quality and safety (Kristiansen et al., 2020).
However, in order to get a better understanding of the physiology and spoilage potential of spore formers, a more extensive knowledge about their metabolic pathways and proteolytic activity is required.

2. Materials and methods.

2.1. Overall experiment.

Growth and proteolytic activity of several bacterial isolates belonging to the *Bacillus cereus* group and to the *Clostridium sensu strictu* cluster, together with two *Staphylococcus epidermidis* isolates, were investigated in UHT milk.

Growth, proteolytic activity and metabolic productions of one *Bacillus cereus* isolate, one *Clostridium tyrobutyricum* isolate and one *C. sporogenes / botulinum* isolate where further investigated in a cheese model.

An overview of the practical's structure is given in Figure 8.

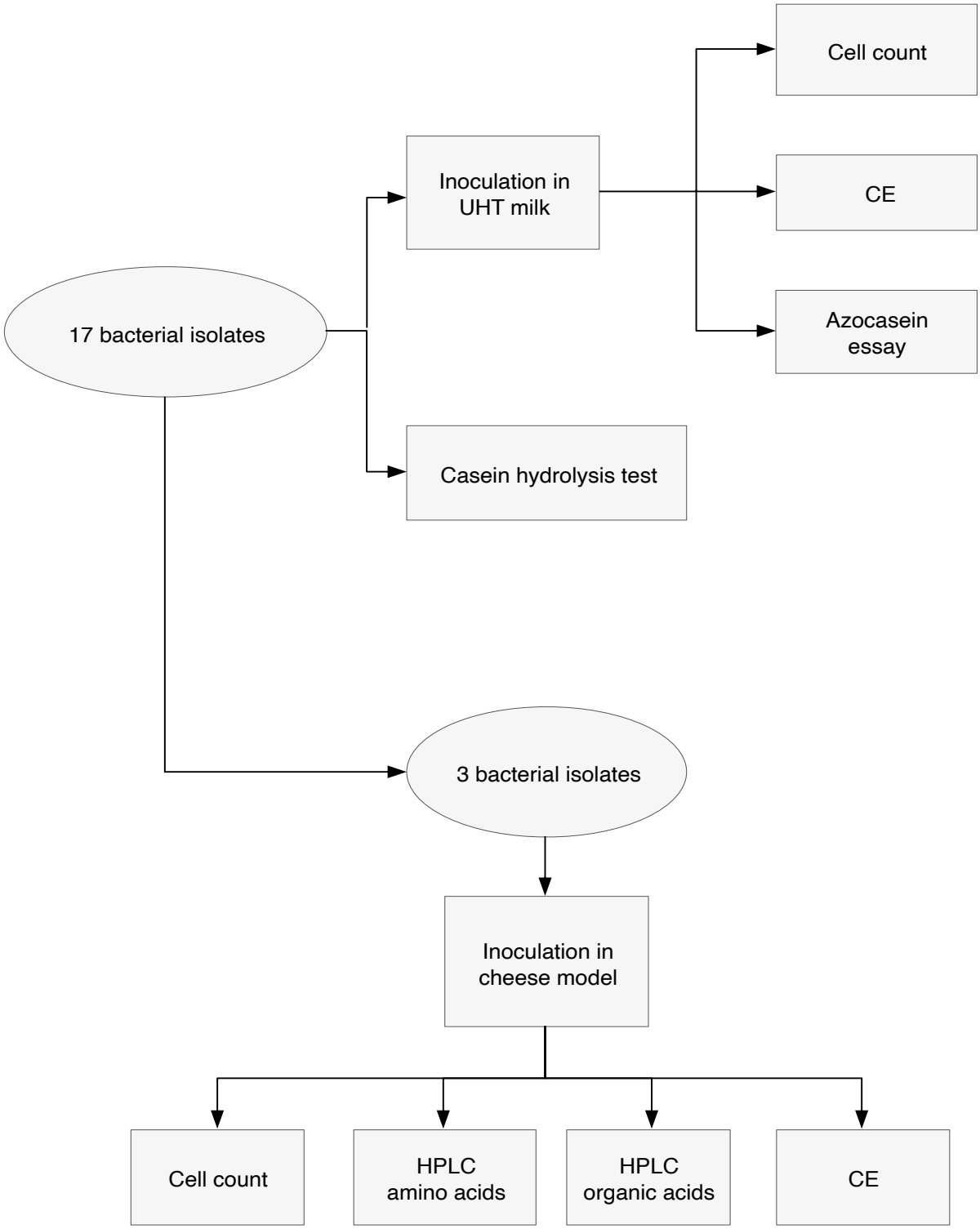


Figure 8. Flow chart of the project.

All the isolates were tested for their casein degradation ability on skim milk agar. Capillary electrophoresis (CE) was used to provide casein degradations patterns in UHT milk and in the

cheese model. Extracellular protease activity was determined in UHT milk by performing an azocasein assay, while metabolism in the cheese model, in terms of free amino acids, organic acids and carbohydrates, was analyzed using High Pressure Liquid Chromatography (HPLC). As the accessibility to the laboratories was reduced due to the Corona situation, determination of cell concentrations with Bürker counting chamber was used instead of plate counting to investigate bacterial growth.

The bacterial isolates used in the tests (Table 2) were previously obtained from raw milk, skimmed milk, pasteurized skimmed milk and retentate along the processing line for milk powders during a previous master thesis (Kristiansen et al., 2020). The isolates were identified by sequencing the 16S RNA gene by Kristiansen (2020).

Table 2. Bacterial isolates, with relative codes, inoculated in UHT milk and in the cheese model and tested for casein degradation.

Isolate nr	Isolate	Casein degradation test	UHT milk	Cheese model
137	***Staphylococcus epidermidis***	+	+	-
137*	***Staphylococcus epidermidis***	+	+	-
22	***Bacillus thuringiensis/cereus/tropicus/wiedmanni***	+	+	-
22*	***Bacillus thuringiensis/toyonensis/vereus/paramycoides***	+	+	-
31	***Bacillus wiedmanni/cereus/anthracis***	+	+	+
31*	***Bacillus cereus***	+	+	-
55	***Bacillus proteolyticus/cereus***	+	+	-
56	***Bacillus cereus***	+	+	-
17	***Clostridium diolis/ beijerinckii***	+	+	-
23	***Clostridium diolis/beijerinckii***	+	+	-
19	***Clostridium tyrobutyricum***	+	+	-
24	***Clostridium tyrobutyricum***	+	+	-
16	***Clostridium tyrobutyricum***	+	+	-
138	***Clostridium tyrobutyricum***	+	+	+
139	***Clostridium tyrobutyricum***	-	-	-
53	***Clostridium sporogenes/ botulinum***	+	+	-
54	***Clostridium sporogenes/botulinum***	+	+	+

*Isolated in aerobic conditions.

Isolate 31 will be further referred to as *Bacillus wiedmanni/cereus*, while isolates 53 and 54 will be mentioned as *Clostridium sporogenes*. The handling of the bacterial isolates was performed under sterile conditions throughout the project.

2.2. Preparation of the media for bacterial cultivation.

Brain Heart Infusion (BHI) and Reinforced Clostridia Medium (RCM) broths were used as media for bacterial cultivation, BHI for *Bacillus* ssp. and staphylococci and RCM for clostridia. BHI broth was made with 37 g/L BHI powder (Oxoid Ltd, UK) and RCM broth with 38 g/L RCM powder (Oxoid Ltd, UK). The mixtures were kept in a warm bath at ca 100 °C until powder dissolution before being autoclaved in 10 ml glass tubes at 121 ºC for 20 minutes.

2.3. Bacterial cultures revival.

After been stored at -80 ºC, the frozen isolates were each inoculated into 10 ml broth, Bacilli and Streptococci in BHI broth and Clostridia in RCM broth, respectively. The cultures were then incubated under anaerobic conditions at 37 ºC for three days. In order to guarantee consistent optimal growth and metabolic activity at the time of utilization, bacterial cultures were continuously inoculated (weekly) all along the trial period inoculating 100 µl culture in new broth.

2.4. Casein degradation test.

A casein degradation test was performed using skim milk agar, adjusting a protocol described elsewhere (Pailin et al., 2001). Skim milk agar was made with 20 g/L Plate Count Skim Milk Agar Powder (Merck KGaA, Darmstadt, Germany) / L. The mixture was let to rest for about 15 min at room temperature and then kept in a warm bath at ca 100 °C under occasional stirring until powder dissolution, autoclaved at 121 ºC for 15 minutes, poured into plates.
Bacterial cultures were inoculated on skim milk agar by spot plating. Previous plating, the plates' surface was dried in a ventilated hood for approximately 40 minutes. Circa 5 µL of undiluted bacterial culture were then inoculated onto the plate on three well-distanced spots. One plate was used for each bacterial culture. Once the spots dried up, the plates were incubated at 37 ºC for 24 hours. Casein degradation was determined visually, as it resulted in clearing zones around the colonies. An example of casein degradation on skimmed milk agar is given in Figure 9.

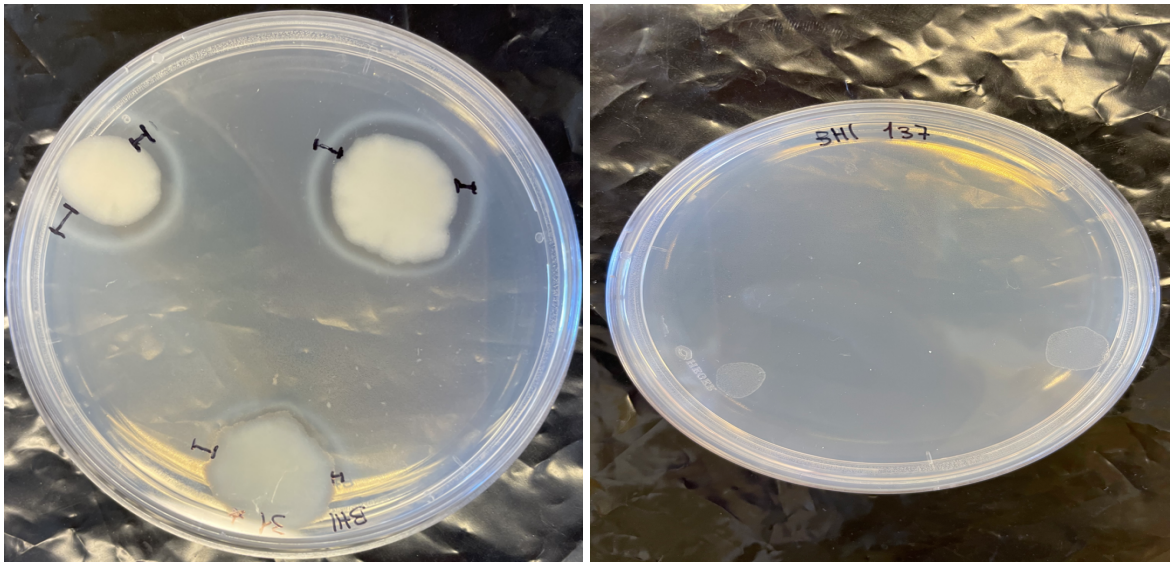


Figure 9. Casein degradation resulting in clearing zones around the bacterial colonies (left) and a negative casein degradation test (right).

In case of a positive result, the clearing zone diameter was measured for all the three colonies and an average was calculated.

Those plates that did not show colony formation after 24 h were further incubated at 37 ºC for three days and observed again.

2.5. Growth and proteolytic activity in UHT milk.

2.5.1. UHT milk trial.

An overview of the tests performed on UHT milk is given in Figure 10.

A sample set (set A) of UHT milk (TINE, Oslo, Norway) of a volume of 10 ml was inoculated with 100 μL bacterial culture and then incubated at 37 ºC for 24 hours, after which the Azocasein assay, cell count and CE were performed. The milk samples were then further incubated at 37 ºC for another 3 days and a new set of analysis was performed. As these last analysis gave somehow incongruous results, a new set of samples (set B) was incubated for four days before sampling.

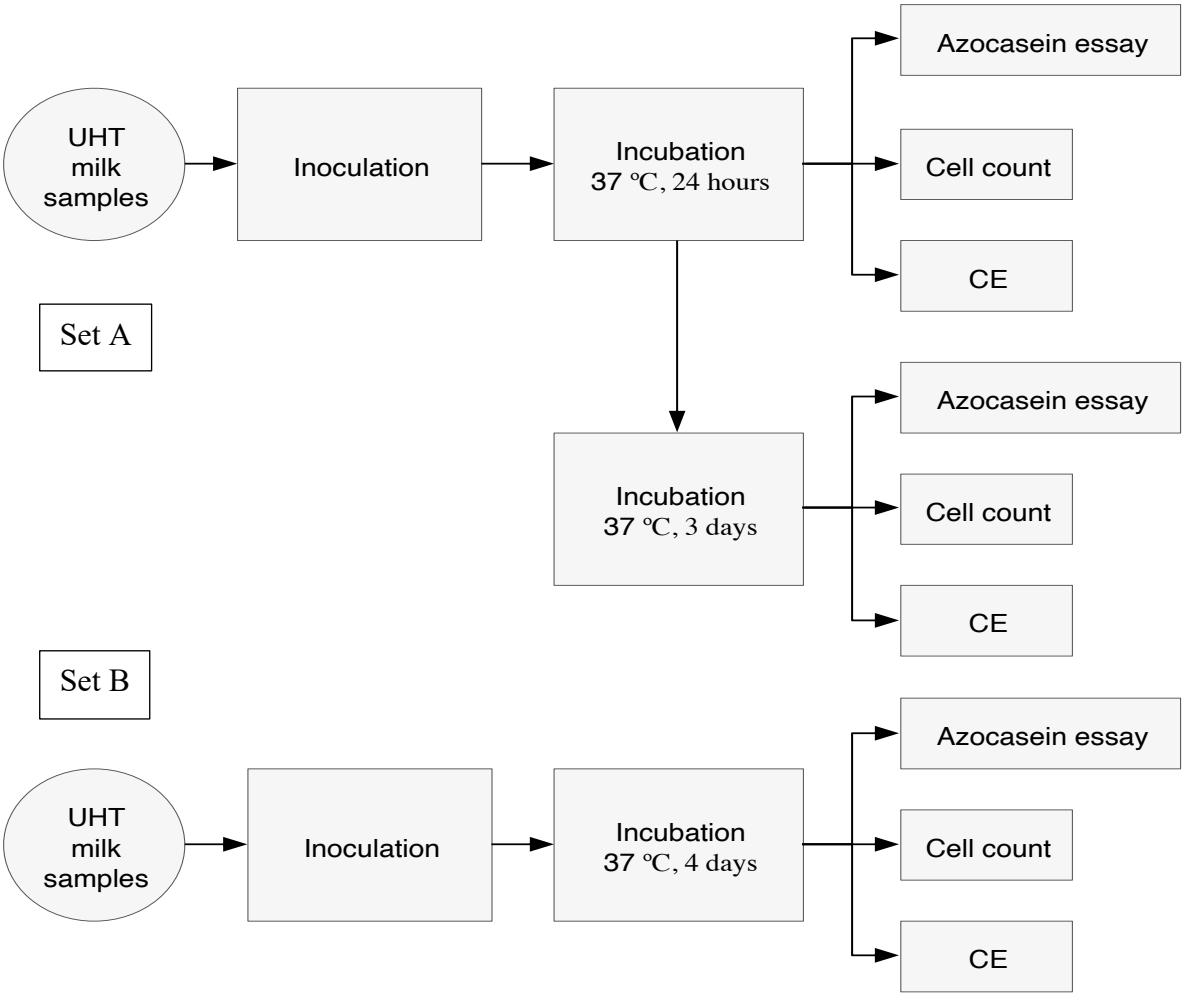


Figure 10. Experimental design for the UHT milk trial.

2.5.2. Azocasein essay.

Adjusting a protocol described elsewhere (Andreani et al., 2016), Azocasein assay was performed using a 3% stock solution of Azocasein 2756 (Sigma-Aldrich, USA) in 100 mM Tris Buffer containing 5mM of $CaCl_2$. Tris buffer's pH was adjusted to 8.5 using 1 M HCL solution. Prior Azocasein addition, the Tris Buffer was warmed up to 50 ºC and then cooled down to 37 ºC.

After incubation, the UHT milk samples were centrifugated at 20000 g. Of the cell free supernatant obtained, 100 µL were added to 100 µL Azocasein stock solution and 300 µL 50 mM Na_2HPO_4, pH 7.5. The samples were incubated at 37 ºC for 1 hour, then added 500 µL of 20% solution of trichloroacetic acid (TCA), which stopped the reaction. The samples were then centrifugated at 12000 g for 10 minutes and the absorbance of the supernatant was read with a spectrophotometer (Genesis 50 UV-visible spectrophotometer, Thermo Fisher Scientific Inc.). The spectrophotometer was blanked with a negative control consisting in 100 µL not-inoculated UHT milk, 100 µL Azocasein stock solution and 300 µL 50mM Na2HPO4, pH 7.5, added 500 µL of 20% TCA solution without previous incubation.

For each Azocasein assay, references with relative control samples were made with distilled water and proteinase K using the same procedure as described above, where 100 µL water and 100 µL caseinate respectively substituted the cell free supernatant from the milk samples.

2.5.3. Cell count and determination of cell number.

Previous cell count, cell washing was performed. A volume of 1 mL milk sample was centrifugated at 20000 g for three minutes (Centrifuge 5424 R, Eppendorf AG). The pellet was then resuspended in 1 mL Ringer's solution. The procedure was performed twice.

Cell count was performed under the light microscope. A Bürker counting chamber was used as described elsewhere (Gunetti et al., 2012). A tenfold dilution series of the washed sample was made with Ringer's solution. From the different dilutions, ca 10 µL were transferred onto the Bürker counting chamber and observed under the microscope at 40x magnification. After calculating the average of the cells counted in four of the counting chamber's squares, the cell number pr. mL milk sample was determined as described in (1):

$$cells\ pr.square\ x\ cubic\ volume\ x\ dilution\ factor \quad (1)$$

2.5.4. Sample preparation for capillary electrophoresis (CE).

Adjusting a protocol described elsewhere (Nilsson et al., 2020), samples for capillary electrophoresis were prepared by adding 600 μL milk sample to 900 μL sample buffer. The mixture was let to rest for one hour, then centrifugated at low speed for 2 minutes and filtered with a 0.2 μm cellulose acetate filter.

The filtrated sample (50 μL) was run on a capillary electrochromatograph (Agilent 7100 CE System, Agilent Technologies Inc.) by laboratory personnel, as described by Nilsson et al. (2020).

2.6. Growth, proteolytic activity and metabolic productions in the cheese model.

2.6.1. Cheese model preparation.

The cheese used to prepare the cheese model was manufactured at the Institute's Pilot Plant in October 2020 within a practical exercise in the NMBU course MVI383A, Dairy Technology. Cheese manufacture followed the steps common for Gouda cheese production (McSweeney et al., 2017). However, the cheese used for the preparation of the cheese model was frozen after 24 hours from the start of the manufacture and did not undergo brine salting. A flow chart of the cheese manufacture is given in Figure 11.

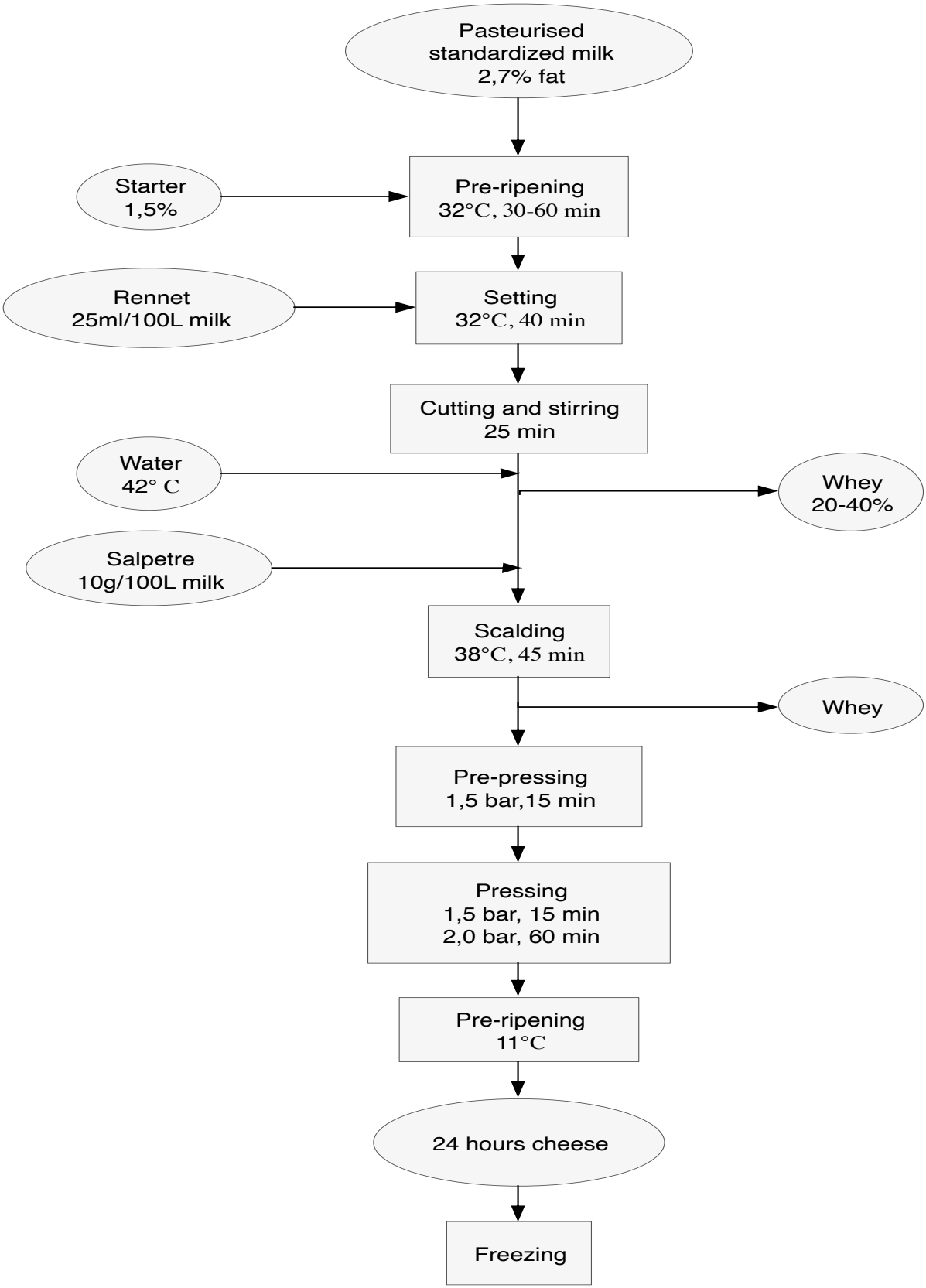


Figure 11. Flow chart for manufacture of the cheese used for the preparation of the cheese model.

Pasteurized milk with a fat content of 2,7% was added a starter concentration of 1,5%. After a pre-ripening of 60 minutes duration at 32 ºC, rennet was added in a concentration of 25mL /100 L milk.

Setting took place at 32 ºC and had lasted 40 minutes. After cutting, the curd was stirred for 25 minutes. Water at 42 ºC was added after the first whey drainage and salpetre addition (10 g / 100 L milk). After scalding at 38 ºC for 45 minutes and a second whey drainage, the cheese underwent pre-pressing under 1,5 bar for 15 minutes. Pressing was performed at 1,5 bar for the first 15 minutes and then at 2,0 bar for 1 hour. The cheese was then frozen after overnight storage at 11 ºC.

Previous preparation of the cheese model, the cheese was thawed overnight at 4 °C and then shredded with a grinding machine. Samples were collected for pH measurement and for determination of dry matter (DM), of protein distribution by CE and of carbohydrates, organic acids and amino acids concentrations by HPLC.

To make the cheese model, 11 g of shredded cheese were placed in heat-resistant plastic bags. The bags were vacuum-sealed and then sterilized by autoclaving at 121 ºC for 20 minutes. They were then stored at 4°C until inoculation.

2.6.2. Determination of dry matter.

In order to keep water evaporation to a minimum, samples for determination of dry matter. were handled straight after shredding. Cheese dry matter was determined according to IDF Standard 4-2004 (No, 2004).

Three parallels of ca 5 g shredded cheese were weighted together with the cups to be dried in. The cheese was left to dry overnight at room temperature and further dried in a ventilated drying cabinet at 102 ± 1 ºC for 20 hours. The cheese samples were weighted again with their cups and cheese dry matter content was determined as in (2). An average of the three parallels was then calculated.

$$dry\ matter\ \% = \frac{dry\ sample\ weight}{sample\ weight}\ x100 \qquad (2)$$

2.6.3. Ph measurement.

One sample of 25.0 g shredded cheese was mixed with 10 mL distilled water. After a resting period of 30 minutes pH was measured with a calibrated pH-meter (Radiometer PHM 92 Lab pH meter, Radiometer Medical ApS).

2.6.4. Structure of the cheese model trial and analysis set.

Bacillus wiedmannii / cereus 31, *Clostridium tyrobutyricum* 138 and *C. sporogenes/ botulinum* 54 were selected for inoculation into the cheese model.

The experimental factors for this part of the project were, in addition to bacterial species, salt concentration and incubation. Analysis of carbohydrates, organic acids and free amino acids by HPLC were run on unsterilized cheese samples with 0 %. and 2.0% salt addition.

Each bacterial culture was inoculated in plastic bags with unsalted cheese model (0% salt) and with cheese model added 2.0 % (w/w) salt. Cell number determination, CE and HPLC for analysis of free amino acids and organic acids were performed at the time of inoculation (Time 0) and after one week of incubation at 37 °C (Time 1). Bacterial growth and gas formation were visually controlled along the incubation period. An overview of the practical's structure is given in Figure 12.

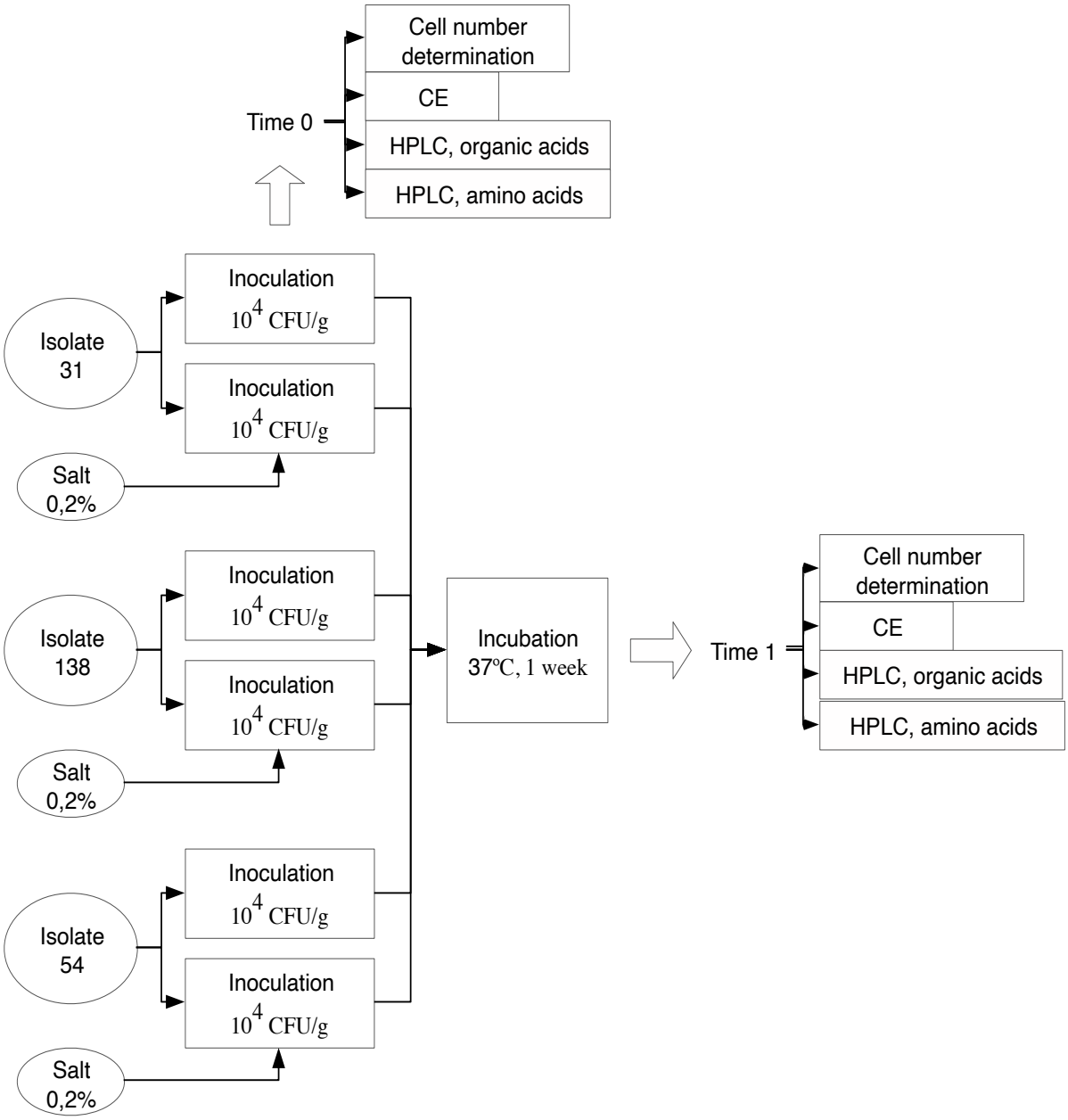


Figure 12. Flow chart of the cheese model trial and set of analysis performed on cheese samples at inoculation (Time 0) and after one week incubation at 37°C (Time 1).

2.6.5. Cheese model inoculation.

Before inoculation, the bacterial cultures were washed from the broth and tenfold dilution series were made with Ringer's solution, as described in paragraph 2.5.3. The cheese bags were inoculated with a cell concentration of 10^4 CFU/g, after cell counting in Bürker's counting chamber and cell number determination (see paragraph 2.5.3). The bags were cut open and the bacterial culture was pipetted inside, and manually mixed into the cheese to achieve a bacterial distribution as homogeneous as possible. Salt, in form of dry natrium chloride, was also

manually mixed into the cheese. The bags were then vacuum sealed again and incubated at 37°C for one week. All samples were inoculated in triplicates.

2.6.6. Cell count and determination of cell number in cheese bags.

The cheese was transferred from the bag to a sterile Omni-Mixer Homogenizer (OMNI, Kennesaw, USA) and was homogenized with 99 mL 2% (w/V) citrate water for two minutes at speed 4.

The cheese emulsion was further diluted with Ringer's water in a tenfold dilution series, and an attempt of cell counting with Bürker counting chamber was made as described in paragraph 2.5.3. As the massive presence of impurities from the cheese made cell counting impractical, the cheese emulsion was centrifugated at 2000 g for one minute to remove the heaviest particles and then cell washing was performed following the procedure described in paragraph 2.5.3. After cell counting with Bürker counting chamber, cell number/ g cheese was determined as described in paragraph 2.5.3.

2.6.7. Samples preparation for capillary electrophoresis (CE).

Samples for CE were prepared from 600 μL of the cheese emulsion (see paragraph 2.5.4), following the procedure described in paragraph 2.5.4. CE was run with a capillary electrochromatograph as described in paragraph 2.5.4.

2.6.8. Sample preparation for High Performance Liquid Chromatography (HPLC) for analysis of carbohydrates and organic acids.

Adjusting a protocol described elsewhere (Narvhus et al., 1998), samples of unsterilized cheese (2 g) were added 5 mL Milli-Q water (Milli-Q Direct Water Purification System, Merck Millipore, Merck Group), 0,7 mL 0,5 M H_2OSO_4 and 20 mL CH_3CN. The samples were first shaken by hand for ca one minute and then by a rotator mixer for 30 minutes. The samples were then centrifugated in a Gerber centrifuge at 7000 rpm for 15 minutes. The supernatant was finally filtrated with a 0,2 μm filter into a HPLC vial. Twenty-five μL of the filtrate were then analyzed using the HPLC (Perkin Elmer, USA) by laboratory personnel, as described elsewhere (Moe et al., 2013). As for the sterilized and inoculated cheese, the content of the bags was shredded with a grinding machine before further sample preparation was performed as described above.

2.6.9. Sample preparation for High Performance Liquid Chromatography (HPLC) for analysis of free amino acids.

Adjusting a protocol described elsewhere (Kabelova et al., 2009), samples of unsterilized cheese (1.5 g) were mixed with 15 mL 0,4 μmol/mL L-norvaline in 0,1 M HCL in Ultraturrax (IKA -Werke GmbH & Co., Germany) at 20000 rpm for 5 minutes. The mixture underwent ultrasound bath for 30 minutes and then centrifugation at 3400 g for 40 minutes at 4°C. Of the supernatant, 800 μL were mixed with 800 μL 4% TCA on a minishaker. After resting in an ice bath for 30 minutes, the samples were centrifugated at 15600 g for 5 minutes at 4°C. The supernatant was finally filtrated with a 0,2 μm filter into a HPLC vial and 50 μL of the filtrate was run on a HPLC (Perkin Elmer, USA) by laboratory personnel, as described elsewhere (Moe et al., 2013). Sterilized and inoculated cheese was shredded with a grinding machine and samples for HPLC were prepared as described above.

2.7. Statistical analysis.

The significance of the experimental factors and their interactions for cell concentration and for the levels of free amino acids, carbohydrates and organic acids obtained in the cheese bags was determined by Two Ways Analysis of Variance (ANOVA). The statistical analysis was performed using Minitab statistical software (Minitab, LLC).

3. Results.

3.1. Casein degradation test.

The casein degradation test showed the isolates' ability to produce casein degrading enzymes, which resulted in casein hydrolysis and in the formation of clear zones around the colonies. Observations of colony and zone formation on skim milk agar after 24 hours incubation at 37°C are reported in Table 3.

Table 3. Growth and casein degradation of the isolates in skim milk agar. Where casein degradation took place, the average diameter for the clear zones observed around the bacterial colonies is given in mm.

Isolate nr	Isolate	Growth	Clear zone diameter (mm)
137	*Staphylococcus epidermidis*	+	-
137*	*Staphylococcus epidermidis*	+	2.8
22	*Bacillus thuringiensis/cereus/tropicus/wiedmanni*	+	2.0
22*	*Bacillusthuringiensis/toyonensis/cereus/paramycoides*	+	3.3
31	*Bacillus wiedmanni/cereus*	+	2.3
31*	*Bacillus cereus*	+	4.3
55	*Bacillus proteolyticus/cereus*	+	2.0
56	*Bacillus cereus*	+	2.8
17	*Clostridium diolis/ beijerinckii*	-	-
23	*Clostridium diolis/beijerinckii*	+	-
19	*Clostridium tyrobutyricum*	+	3.7
24	*Clostridium tyrobutyricum*	-	-
16	*Clostridium tyrobutyricum*	-	-
138	*Clostridium tyrobutyricum*	-	-
139	*Clostridium tyrobutyricum*	-	-
53	*Clostridium sporogenes*	+	3.3
54	*Clostridium sporogenes*	+	4.0

After 24 hours of incubation, all the *Bacillus* ssp. and both isolates of *S. epidermidis* had formed colonies. Clostridial growth was less consistent, with differences among and within the species. Whereas colony formation was observed for both the *C. sporogenes* isolates, only one of the two isolates of *C. diolis/ beijerinckii* was able to grow (*C. diolis/ beijerinckii* 23) and, of *C. tyrobutyricum,* only one isolate out of five (*C. tyrobutyricum* 19).

All the *Bacillus* ssp. tested showed casein degradation ability. Of the two *S. epidermidis* isolates, only *S. epidermidis* 137 formed clear zones. Clostridia showed varying degrading ability, as clear zones were observed for *C. tyrobutyricum* 19 and for both isolates of *C. sporogenes*, but not for *C. diolis/ beijerinckii.*

The average diameter for the clear zones formed by *Bacillus* ssp. ranged between 2 mm (*B. thuringiensis/cereus/tropicus/wiedmanni* 22 and *B. proteolyticus/cereus* 55) and 4.3 mm (*B. wiedmanni/cereus* 31), which were respectively the shortest and the longest average diameters of all the strains tested. Among the clostridia that produced zones, the average diameter ranged between 3.3 mm and 4.0 mm (*C. sporogenes* 53 and 54).

Where no growth was observed after 24 hours, no colonies were formed after further incubation.

3.2. Cell concentration in inoculated UHT milk samples.

A comparison of cell concentrations in UHT milk samples after 24 hours of incubation and four days of incubation is shown in Figure 13. Cell concentrations in sample set A are given for 24 hours of incubation and for four days of incubation, while cell concentrations in sample set B are given only for four days incubation. Cells concentrations of *S. Epidermidis* 137 and of *B. cereus* 56 are given for set B only.

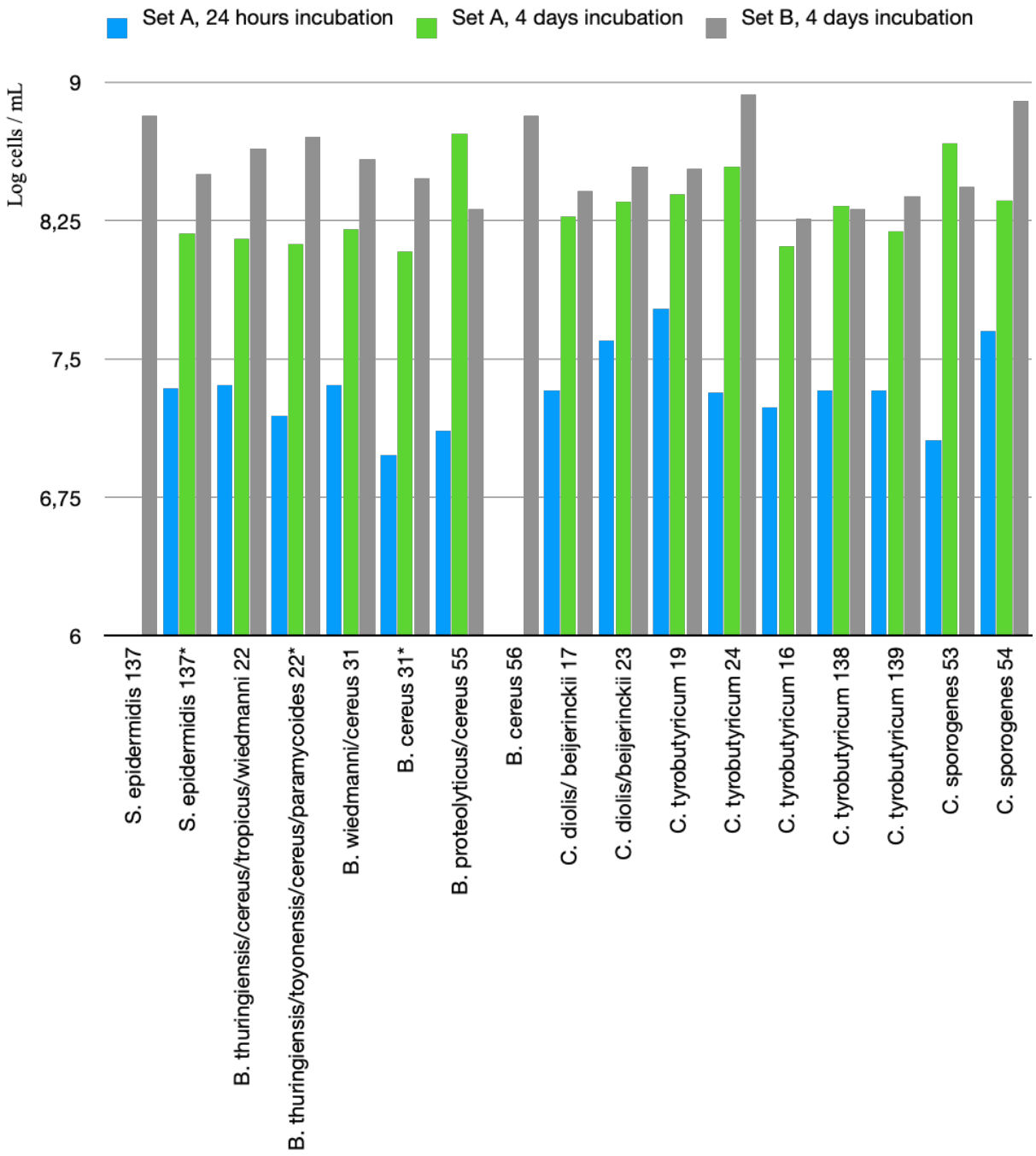


Figure 13. Cell concentration determined in sample set A after 24 hours and four days of incubation and in sample set B after four days of incubation. For each isolate (x axis), cell concentration in set A after 24 hours incubation is given in blue, cell concentration in set A after four days incubation is given in green and cell concentration in set B after four days incubation is given in grey. For all the samples, cell concentration is given in log cells/ mL.

All the samples showed an increase in cell concentration from 24 hours of incubation until four days of incubation. After 24 hours of incubation, most of the isolates had concentrations < 7.5 log cells/mL, whereas *C. diolis/ beijerinckii* 23, *C. tyrobutyricum* 19 and *C. sporogenes* 54

gave 7.60 log cells/mL, 7.77 log cells/mL and 7.65 log cells/mL respectively. After four days incubation a cell concentration > 8.00 log cells/mL was achieved by all the isolates.

After 24 hours incubation, cell concentrations among the investigated *Bacillus* ssp. were slightly lower than those of clostridia. *Bacillus* ssp. concentrations varied between 6.98 log cells/mL (*B. cereus* 31*) and 7.36 cells/mL (*B. wiedmanni/cereus* 31 and *Bacillus thuringiensis /cereus/tropicus/wiedemanni* 22), while clostridial concentration varied between 7.06 log cells/mL (*C. sporogenes* 53) and 7.77 log cells/mL (*C. tyrobutyricum* 19).

Except for isolates *B. proteolyticus/cereus* 55, *C. tyrobutyricum* 138 and *C. sporogenes* 53, cell concentration after four days incubation was somehow lower in set A than in set B. Cell concentration varied between 8.08 log cells/mL (*B. cereus* 31*) and 8.72 (*B. proteolyticus/cereus* 55) in set A and between 8.26 log cells/mL (*C. tyrobutyricum* 16) and 8.93 log cells/mL (*C. tyrobutyricum* 24) in set B.

Regarding set A, minimum difference between cell concentration after 24 hours and cell concentration after four days of incubation was given by *C. tyrobutyricum* 19 and *C. sporogenes* 54, while the largest difference was seen for isolates *B. proteolyticus/cereus* 55 and *C. sporogenes* 53.

3.3. Azocasein assays.

Protease's activity, as measured with the azocasein assay in the inoculated UHT milk samples, is given in absorbance values OD 440 nm in Figure 14. Absorbance of the samples incubated for 24 hours is compared with absorbance of samples incubated for four days, given equal incubation temperature (37°C).

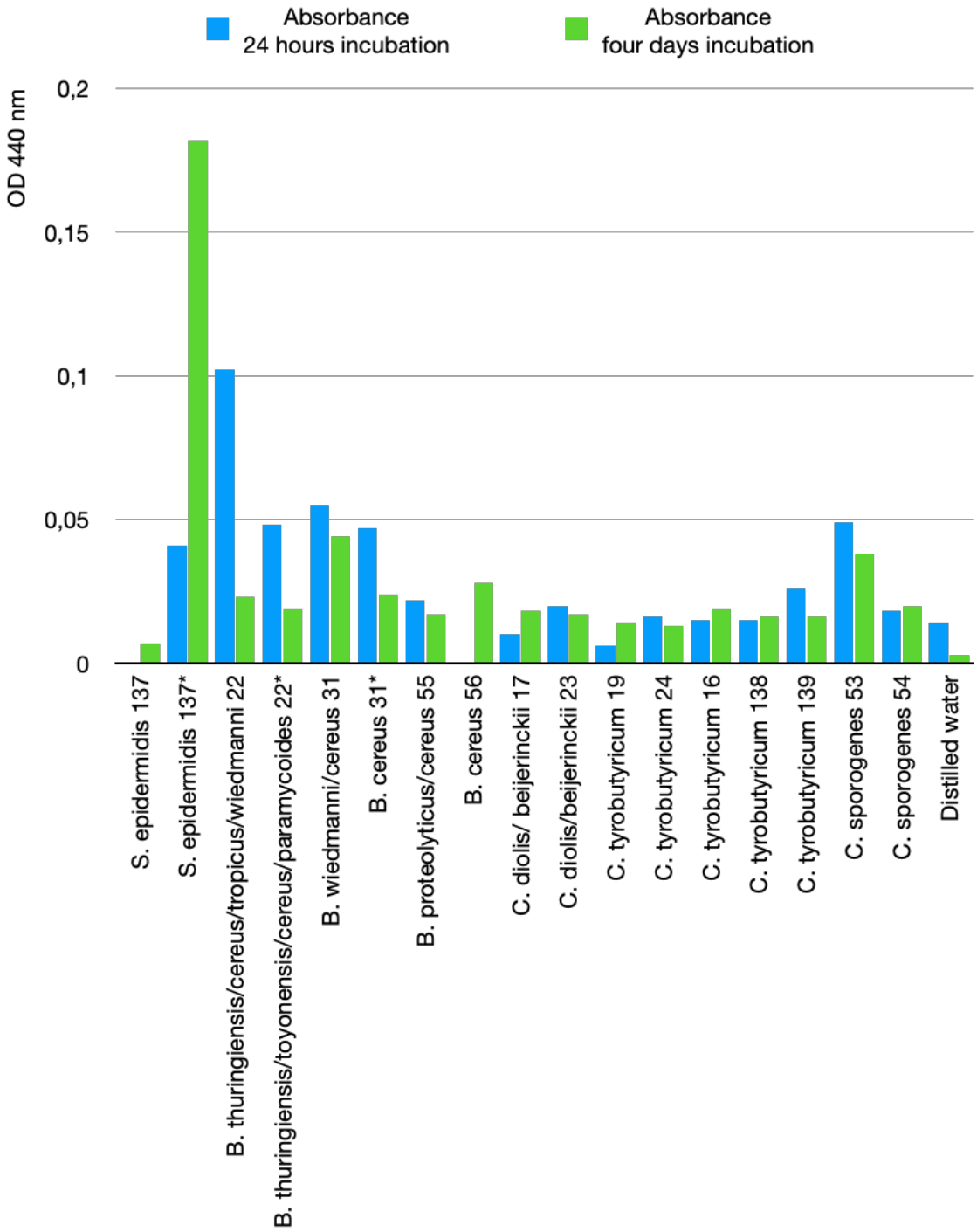


Figure 14. Results of Azocasein assay given in absorbance OD 440 nm after 24 hours of incubation and four days of incubation at 37 °C. For each isolate, absorbance values for 24 hours of incubation are given in blue, while values for four days of incubation are given in green. Values for distilled water are shown as a reference. Absorbance values for proteinase K are not shown.

Proteinase K showed an average absorbance of 1.4010 OD 440 nm. Compared with this reference value, most of the samples showed much lower absorbance, independently from the incubation time.

After 24 hours of incubation, absorbance values > 0.033 OD 440 nm were given only by *B. thuringiensis/cereus/tropicus/wiedemanni* 22 and 22*, *B. wiedmanni/cereus* 31 and *B. cereus* 31* among *Bacillus* ssp., by *C. sporogenes* 53 among clostridia and by *S. Epidermidis* 137. The highest absorbance value given by *Bacillus* ssp. was 0.102 OD 440 nm (*B. thuringiensis/cereus/tropicus/wiedemanni* 22), while the absorbance value given by *C. sporogenes* 53 was 0.049 OD 440 nm.

Of all isolates tested, only *S. Epidermidis* 137* gave higher absorbance after four days incubation than after 24 hours of incubation (0.181 OD 440 and 0.041 OD 440 respectively), while all the other inoculated UHT milk samples showed highest absorbance after 24 hours of incubation.

After four days of incubation, the highest absorbance value was given by *S. Epidermidis* 137* (1.181 OD 440 nm), which was also the highest value measured in the trial.

3.4. Capillary electrophoresis plots for inoculated UHT milk samples.

3.4.1. Results for capillary electrophoresis performed on inoculated UHT milk samples 24 hours incubation at 37°C.

Capillary electrophoresis chromatograms show the degree of degradation of the milk proteins. Milk protein degradation patterns in UHT milk samples incubated with selected isolates for 24 hours at 37°C are given in Figure 15. The isolates shown in the figure were selected as their capillary electrophoresis plots were representative for the proteolytic behavior of the different bacterial species. Isolate 23 was chosen as representative for *C. diolis/ beijerinckii,* B. *wiedmanni/cereus* 31 for *Bacillus* ssp., isolate 54 for *C. sporogenes,* isolate 137 for *S. epidermidis* and isolate 138 for *C. tyrobutyricum.*

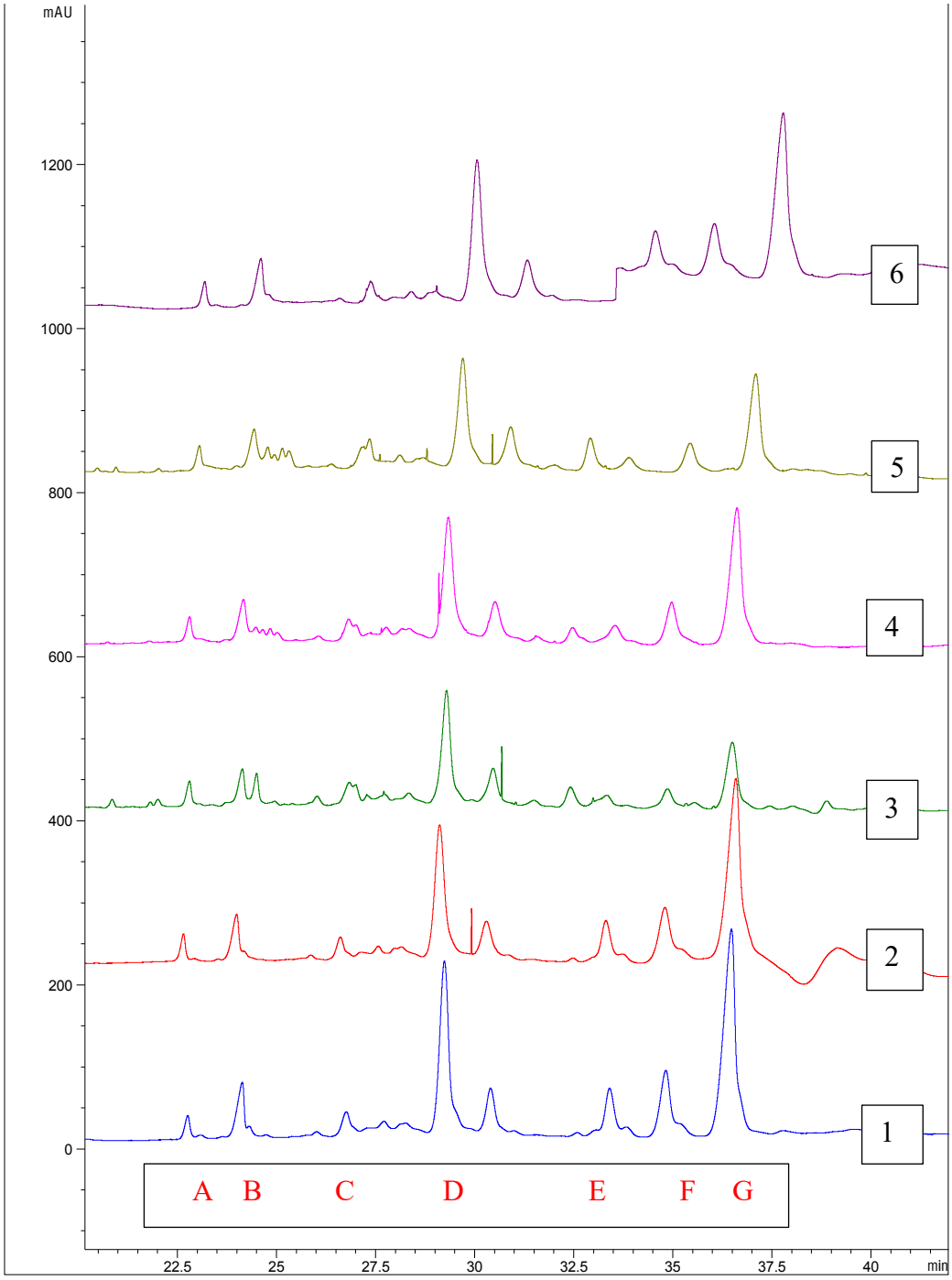


Figure 15. Protein degradation patterns in UHT milk of a selection of isolates after 24 hours incubation at 37°C. From bottom up: CE plot for standard UHT milk (**1**) and proteolytic patterns for *C. diolis/ beijerinckii* 23 (**2**), for B. *wiedmanni/cereus* 31 (**3**), for *C. sporogenes* 54 (**4**), for *S. epidermidis* 137 (**5**) and for *C. tyrobutyricum* 138 (**6**). CE plot for standard UHT milk (**1**) is used here as a reference. The graphs are given by migration time in minutes (x axis) plotted against absorbance in mAU (y axis). In the box at the bottom of the picture letters indicate the position of the different milk proteins, where α-lactalbumin is represented by A, β-lactoglobulin by B, α_{s2} casein by C, α_{s1}casein by D, K casein by E, β_{A1} casein by F and β_{A2} casein by G.

After 24 hours incubation at 37°C, protein degradation patterns differed in α_{s1}casein, (D), of K casein (E), of β_{A1} casein (F) and of β_{A2} casein (G) mainly, while the peaks of α-lactalbumin (A) and α_{s2} casein (C) were quite conserved in all the plots.

Plots for *C. diolis/ beijerinckii* 23 (2) and of *C. tyrobutyricum* 138 (6) were very similar to that for standard UHT milk, with only somehow lower α_{s1}casein peaks.

The plot for the milk sample incubated with *Bacillus* ssp. was the one that differed the most from that for standard UHT milk, and it showed considerably lower peaks of K casein (E), of β_{A1} casein (F) and of β_{A2} casein (G) compared to the proteolytic patterns of all the other strains.

3.4.2. Results for capillary electrophoresis performed on inoculated UHT milk samples after four days incubation at 37°C.

Proteolytic patterns of UHT milk samples incubated with selected isolates for four days at 37°C are shown in Figure 16. *B. thuringiensis/cereus/tropicus/wiedmanni* 22, *B. wiedmanni/cereus* 31 and *B. cereus* 56 were chosen as representative for *Bacillus* ssp., isolate 23 for *C. diolis/ beijerinckii*, isolate 54 for *C. sporogenes* and isolate 138 for *C. tyrobutyricum,* As isolates 137 and 137* showed considerably different proteolytic patterns, they were both used to give an account for *S. epidermidis***.**

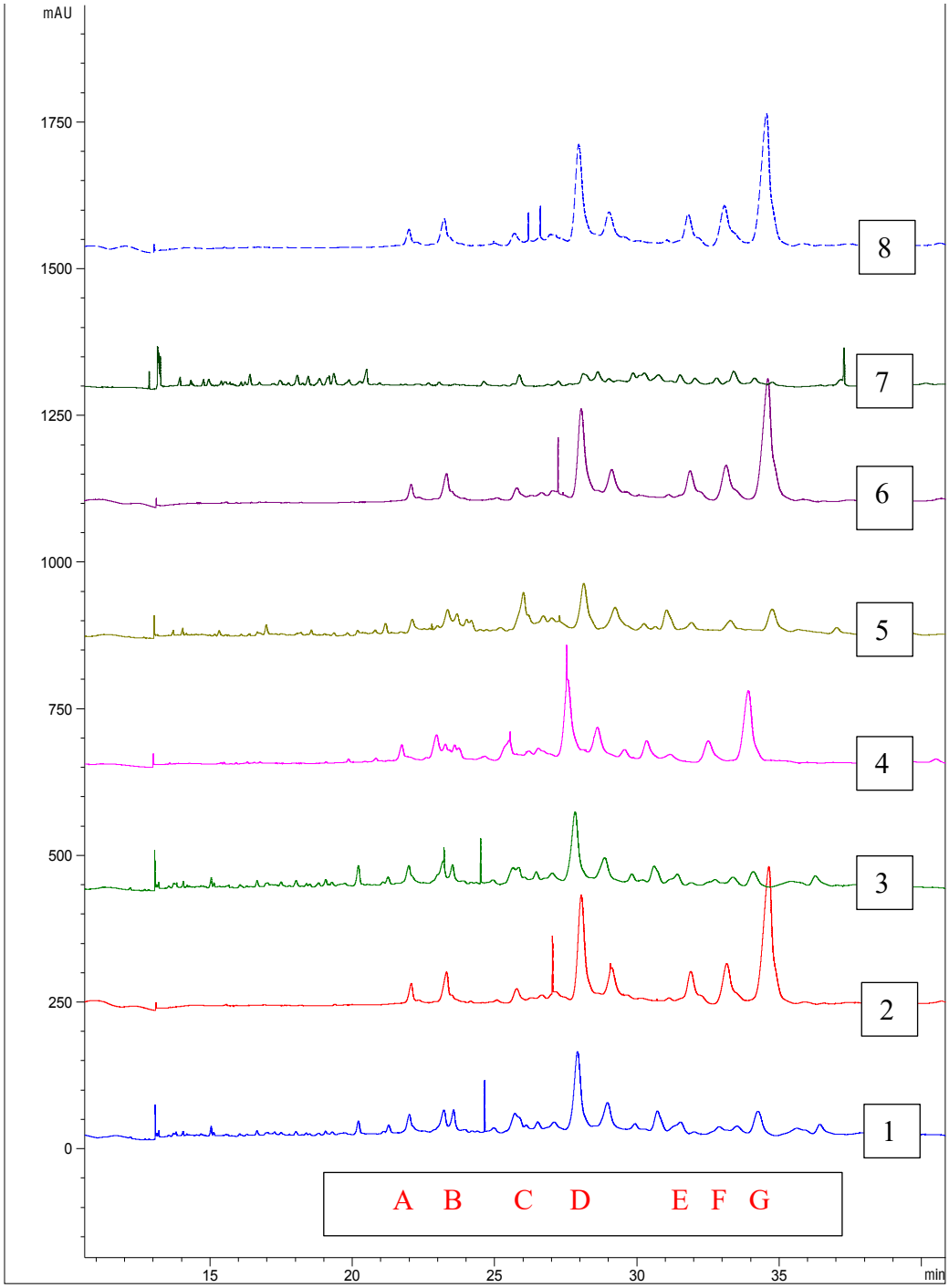


Figure 16. Proteolytic patterns in UHT milk of a selection of isolates after four days incubation at 37°C. From bottom up: proteolytic pattern of *Bacillus thuringiensis/cereus/tropicus/wiedmanni* 22 (**1**), of *C. diolis/ beijerinckii* 23 (**2**), of *Bacillus wiedmanni/cereus* 31 (**3**), of *C. sporogenes* 54 (**4**), of *Bacillus cereus* 56 (**5**), of *S. epidermidis*137 (**6**), *S. epidermidis* 137*(**7**) and of *C. tyrobutyricum* 138 (**8**). Migration time in minutes and absorbance in mAU are given on the x axis and on the y axis respectively. In the box at the bottom of the picture, letters give the indicative position of the different proteins: A represents α-lactalbumin, B β-lactoglobulin, C α_{s2} casein, D α_{s1}casein, E K casein, F β_{A1} casein and G β_{A2} casein.

After four days of incubation at 37°C, UHT milk samples inoculated with *Bacillus* ssp. showed proteolytic patterns where K casein, β_{A1} casein and β_{A2} casein were almost completely degraded, while in the samples inoculated with clostridia hydrolysis of the same proteins were considerably lower. The peaks of α_{s1}casein were also lower for *Bacillus* ssp. than for clostridia.

Among Bacillus ssp., *B. thuringiensis/cereus/tropicus/wiedmanni* 22 (1) and *B. wiedmanni/cereus* 31 (3) showed very similar proteolytic profiles, where the α_{s1}casein and β_{A1} casein peaks were somehow lower for *B. wiedmanni/cereus* 31 (3).
The proteolytic pattern for *B. cereus* 56 (5) showed lower peaks of α-lactalbumin and of α_{s1}casein than the plots for the other *Bacillus* ssp.
Among clostridia, the proteolytic patterns of *C. diolis/ beijerinckii* 23 (2) and of *C. tyrobutyricum* (138 8) were very similar, while the plot for *C. sporogenes* 54 (4) showed lower K casein, β_{A1} casein and β_{A2} casein peaks.

Highest degree of proteolysis was seen in the proteolytic pattern of *S. epidermidis* 137* (7), where all the proteins were degraded almost completely. *S. epidermidis* 137 (8), instead, gave a plot which was very similar to those of clostridia.

3.5. Cheese dry matter (DM) and pH.

The cheese used for the preparation of the cheese model had a DM content of 51.79 % and a pH value of 5.53.

3.6. Capillary electrophoresis (CE) plots for the inoculated cheese model samples after one week incubation (Time 1).

Milk protein degradation patterns showed by *B. wiedmanni/cereus* 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138 in cheese model samples after one week incubation at 37°C are given in Figure 17 for both the cheese salt concentrations (0% and 2% w/w).

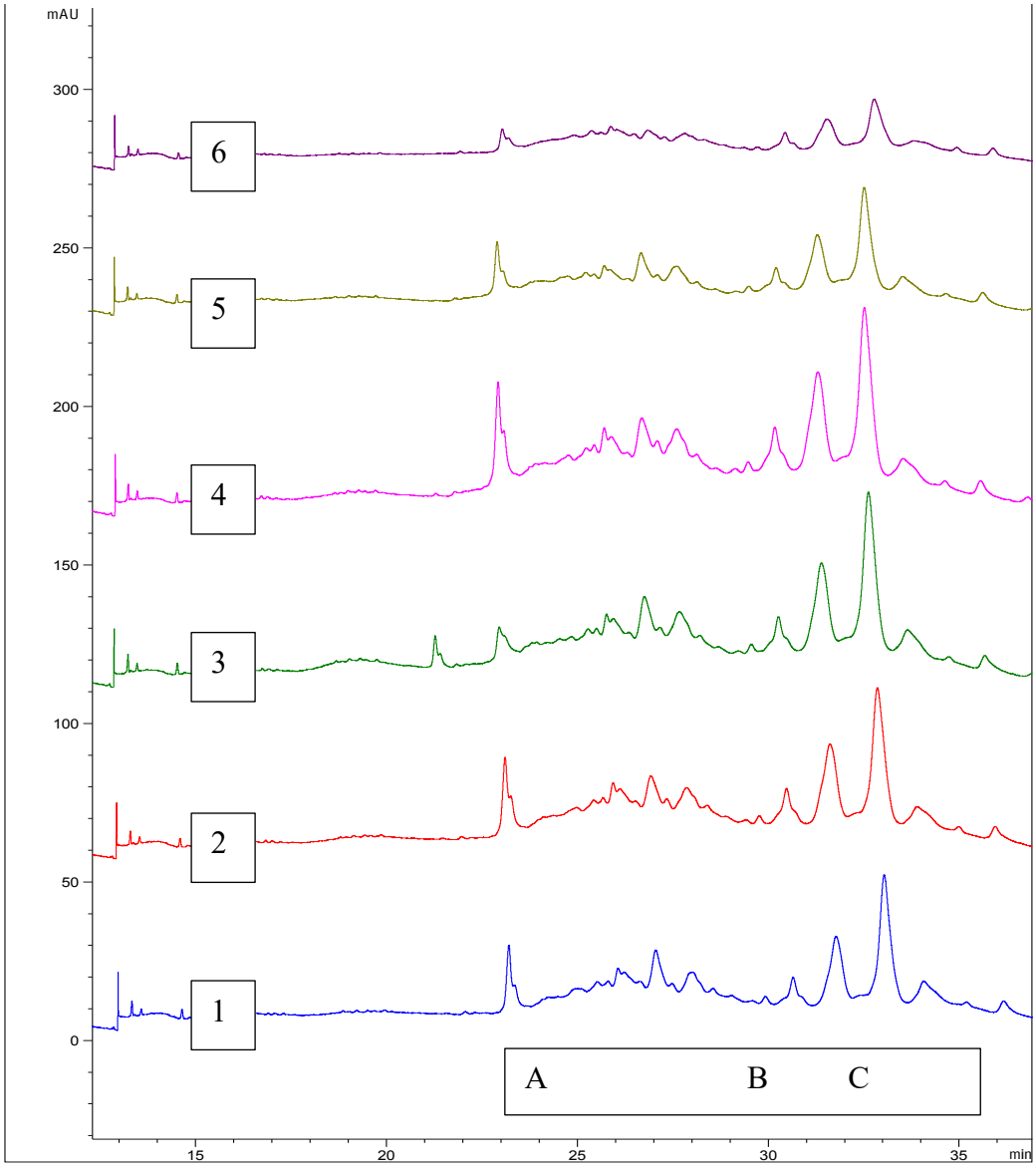


Figure 17. Capillary electrophoresis plots for cheese model samples with different salt concentrations after one week incubation at 37°C (Time 1). CE plots for *C. tyrobutyricum* 138, *Bacillus wiedmanni/cereus* 31 and *C. sporogenes* 54 in samples without salt are given by **(1), (2) and (3)** respectively. CE plots for *C. tyrobutyricum* 138, *Bacillus wiedmanni/cereus* 31 and *C. sporogenes* 54 in samples with 2% salt (w/w) are given by **(4), (5) and (6)** respectively. Migration time in minutes is represented on the x axis and absorbance in mAU on the y axis. In the box at the bottom of the picture, letters give the indicative position of the different proteins: A stays for α_{s2} and α_{s1}casein, B for K casein and C for β_{A1} casein and β_{A2} casein (Otte et al 1997).

At 0 % salt concentration, the CE plots for the three isolates were very similar, showing extended degradation of α_{s2} and α_{s1}casein and of K casein and relatively high β caseins peaks.

The CE plots for cheese model samples incubated with *C. tyrobutyricum* 138 showed a somehow lower degree of proteolysis at 2% salt than at 0% salt, especially in relation to α and β caseins peaks.

B. wiedmanni/cereus 31 and *C. sporogenes* 54, on the other hand, showed higher degree of proteolysis at 2% salt concentration than in the cheese model samples without salt addition.

At 2% salt concentration, the highest degree of casein degradation was seen in the CE plot for *C. sporogenes* 54, where α and K casein peaks were almost completely degraded and β caseins peaks were much lower than those observed in all the other CE plots.

3.7. Cell concentrations in cheese model samples.

3.7.1. Cell concentrations means.

Figure 18 shows the cell concentrations determined in cheese model samples inoculated with *B. wiedmanni/cereus* 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138 at inoculation time (Time 0) and after one week incubation (Time 1) with 0% and 2 % salt concentration. Cell concentrations are represented by means with relative standard error.

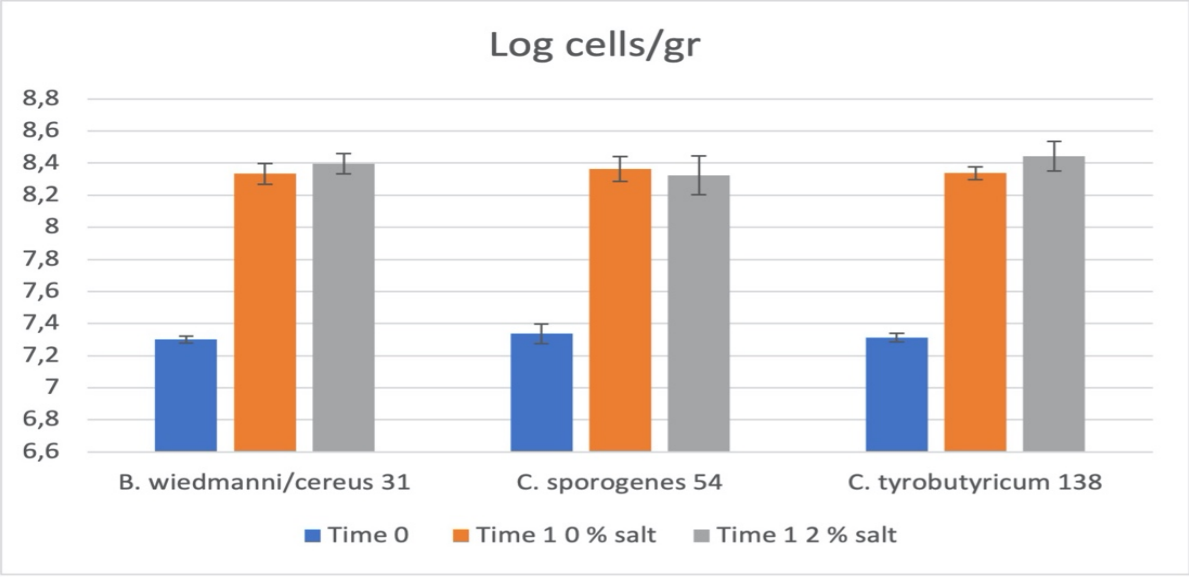


Figure 18. Cell concentrations in cheese model samples at Time 0 and Time 1. Means for cell concentration at Time 0 (blue) are compared with means determined for cheese model samples with 0% and 2% salt addition (orange and grey respectively) incubated with *B.*

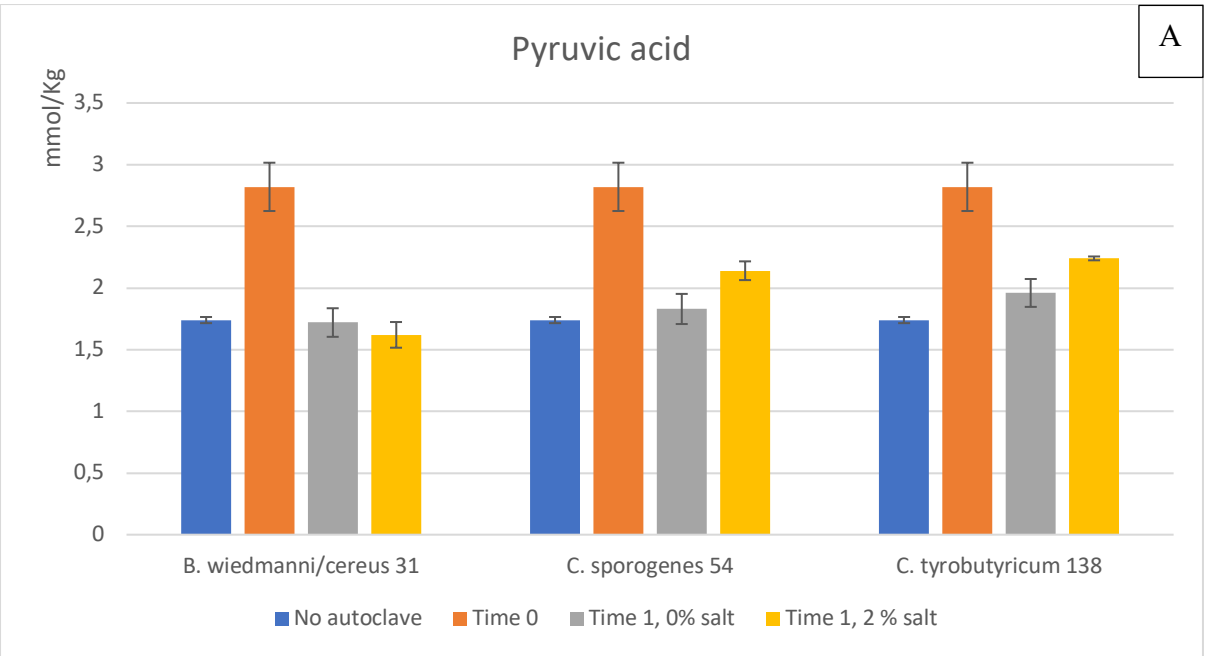
wiedmanni/cereus 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138. Cell concentrations are given in log cells/g. Standard error is given for each cell concentration.

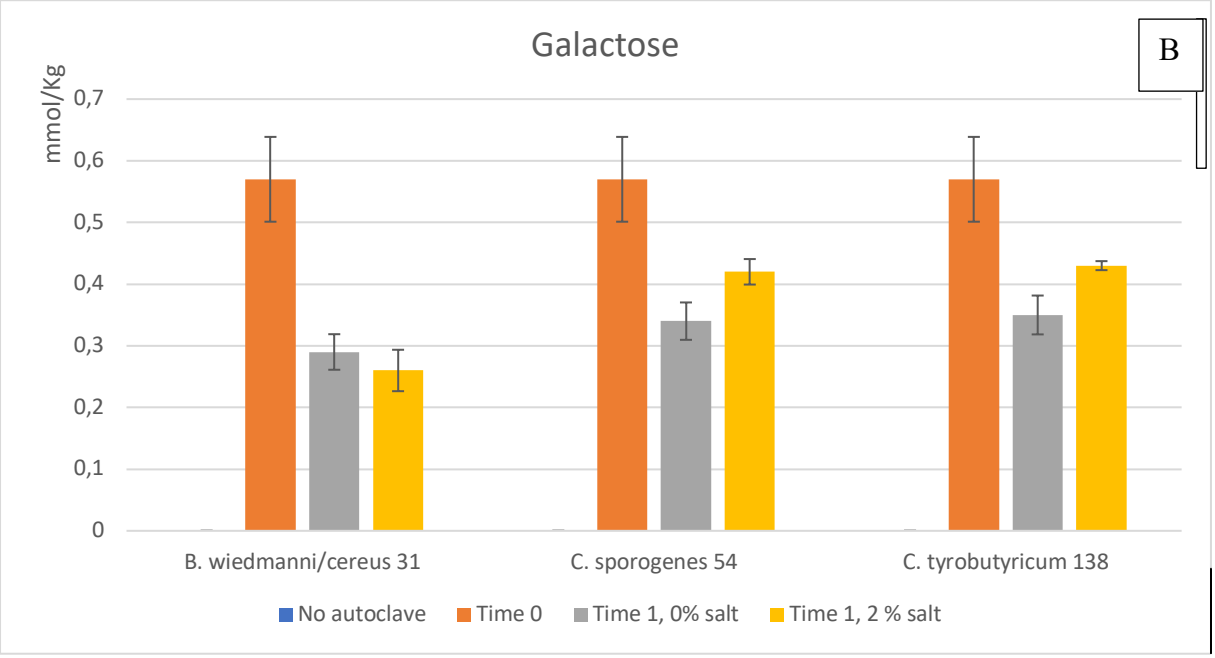
Cell concentration increased with one log during incubation. At Time 1, the means were quite similar for all the samples. Samples incubated with *B. wiedmanni/cereus* and *C. tyrobutyricum* showed s higher cell concentration means at 2% salt concentration, while samples incubated with *C. sporogenes* gave highest mean at 0% salt concentration.
The difference between the analysed sample was larger at Time 0, as shown by a high standard error.
Two ways ANOVA with cell concentration as dependent variable and isolate and salt concentration and time as independent variables showed that only the independent variable time was significant for cell concentration (P value < 0.05).
Time was also the only independent variable to be significant for growth of the three specific isolates (P-values < 0.05).

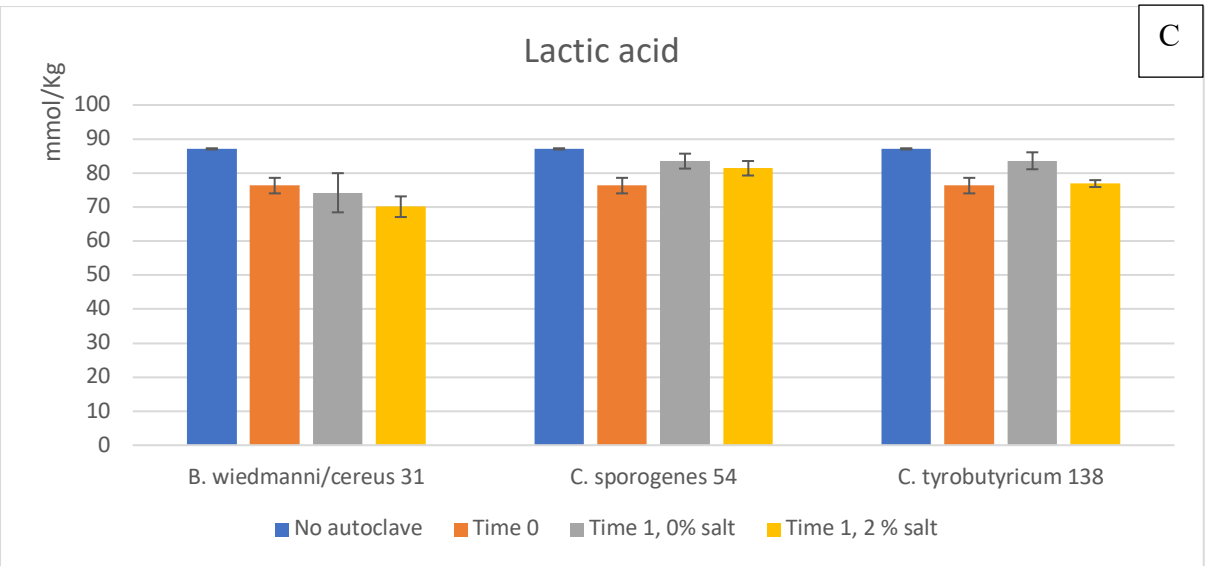
3.8. Carbohydrates and organic acids detected with High Pressure Liquid Chromatography (HPLC) in cheese and cheese model samples.

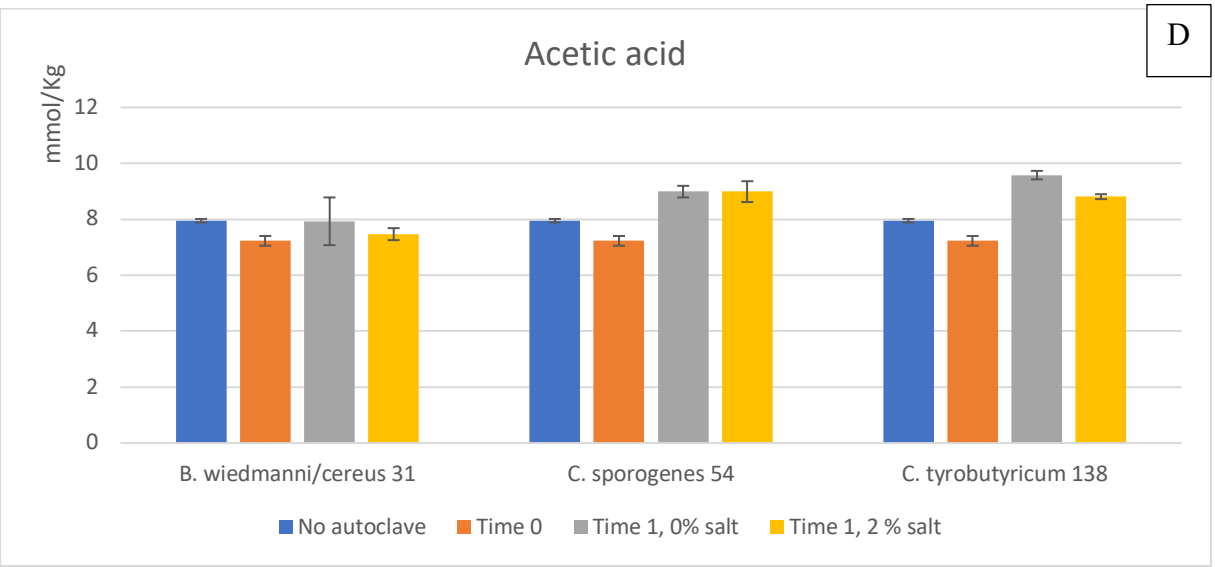
Figure 19 shows the organic acids and carbohydrates concentrations detected in unsterilized cheese samples and in cheese model samples inoculated with *B. wiedmanni/cereus* 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138 at inoculation time (Time 0) and after one week of incubation (Time 1) at 37°C with 0% and 2 % salt concentration.
The organic acids and carbohydrate concentrations are presented as means together with the relative standard error.

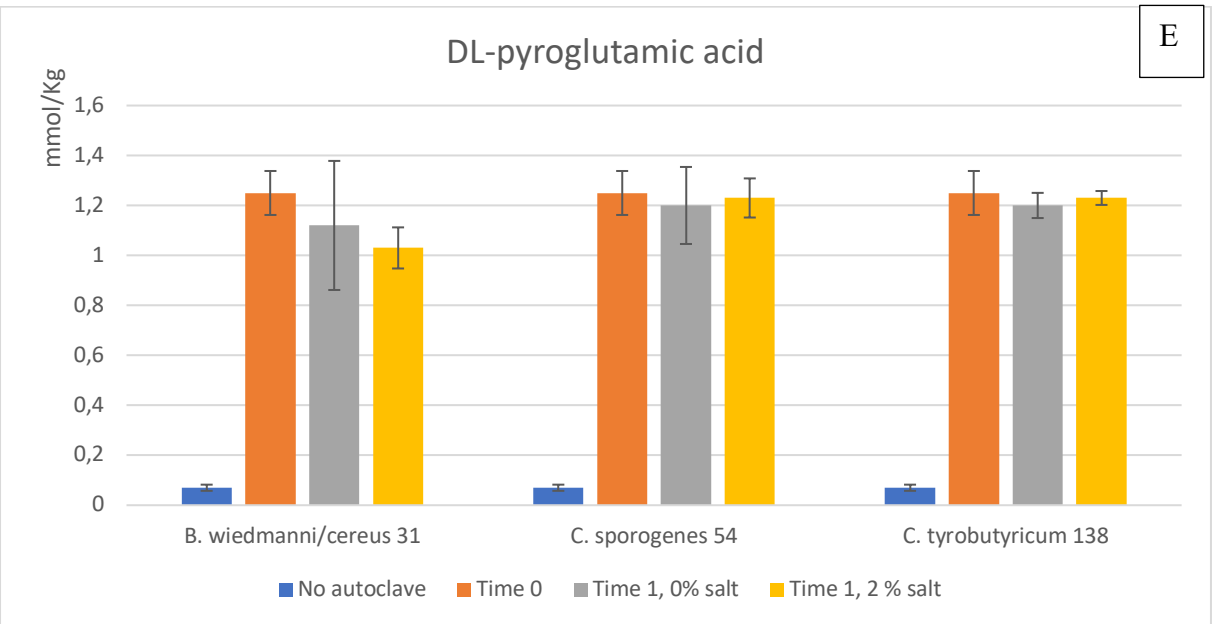
Lactose, citric acid, α-ketoglutaric acid, glucose, succinic and propionic acids were not detected in any of the samples.
Formic acid was detected only in cheese model samples incubated for one week with C. tyrobutyricum. Mean was 0.21 mmol/Kg for samples with 0% salt and 0.20 mmol/Kg for samples with 2% salt.
Orotic and uric acid were detected in trace concentrations and they are therefore not going to be discussed.


A
Pyruvic acid
mmol/Kg
3,5
3
2,5
2
1,5
1
0,5
0
B. wiedmanni/cereus 31
C. sporogenes 54
C. tyrobutyricum 138
No autoclave
Time 0
Time 1, 0% salt
Time 1, 2 % salt


B
Galactose
mmol/Kg
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
B. wiedmanni/cereus 31
C. sporogenes 54
C. tyrobutyricum 138
No autoclave
Time 0
Time 1, 0% salt
Time 1, 2 % salt


C
Lactic acid
mmol/Kg
100
90
80
70
60
50
40
30
20
10
0
B. wiedmanni/cereus 31
C. sporogenes 54
C. tyrobutyricum 138
No autoclave
Time 0
Time 1, 0% salt
Time 1, 2 % salt


D
Acetic acid
mmol/Kg
12
10
8
6
4
2
0
B. wiedmanni/cereus 31
C. sporogenes 54
C. tyrobutyricum 138
No autoclave
Time 0
Time 1, 0% salt
Time 1, 2 % salt


E
DL-pyroglutamic acid
mmol/Kg
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
B. wiedmanni/cereus 31
C. sporogenes 54
C. tyrobutyricum 138
No autoclave
Time 0
Time 1, 0% salt
Time 1, 2 % salt

Figures 19 A – E. Carbohydrates and organic acids concentrations in unsterilized cheese and in cheese model samples at inoculation time and after one week incubation. Plot 19A gives the means for pyruvic acids, plot 19B for galactose, plot 19C for lactic acid, plot D for acetic acid and plot E for DL-pyroglutamic acid. Means for carbohydrates and organic acids concentration in unsterilized cheese (blue) and in cheese model samples at inoculation time (orange) are compared with means determined for cheese model samples with 0% and 2% salt addition (grey and yellow respectively) incubated with *B. wiedmanni/cereus* 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138. Concentrations are given in mmol/Kg. Standard error is given for each concentration.

Unsterilized cheese showed higher means of lactic and acetic acids than cheese model samples, while concentrations of pyruvic acid, galactose and DL-pyroglutamic increased with sterilization.

Pyruvic acid concentration was higher at Time 0 than at Time 1. At Time 1, samples incubated with *C. sporogenes* and *C. tyrobutyricum* gave highest means at 2% salt concentration, while samples incubated with *B. wiedmanni/cereus* showed highest mean at 0% salt. Samples incubated with clostridia gave highest means for both salt concentrations.
The difference between the analysed sample was largest at Time 0, as shown by a high standard error.

Galactose was not detected in unsterilized cheese samples, and it was higher in concentration at Time 0 than at Time 1.
At Time 1, samples incubated with *C. sporogenes* and *C. tyrobutyricum* showed higher means at 2% salt concentration, while samples incubated with *B. wiedmanni/cereus* gave highest mean at 0% salt. Samples incubated with *C. tyrobutyricum* gave highest means for both salt concentrations, while samples incubated with *B. wiedmanni/cereus* gave lowest means. The difference between the analysed sample was largest at Time 0, as shown by a high standard error.

Lactic acid was the most abundant organic acid in all the samples, with concentrations ranging between 6000 and 8000 ppm.

Lactic acid concentration was somehow higher at Time 0 than at Time 1 for samples incubated with *B. wiedmanni/cereus*, while samples incubated with clostridia showed highest means at Time 1, 0% salt.
Samples incubated with *B. wiedmanni/cereus*, no salt gave the largest difference, as shown by a high standard error.

Acetic acid means were higher at Time 1 than at Time 0. At Time 1, samples with no salt addition gave somehow higher means than samples with 2% salt. Highest means were given by samples incubated with clostridia.
Samples incubated with *B. wiedmanni/cereus*, no salt gave the largest difference, as shown by a high standard error.

DL- pyroglutamic acid concentration was higher at Time 0 than at Time 1. At time 1, means for samples incubated with *B. wiedmanni/cereus* were the lowest. Samples incubated with clostridia showed highest means at 2% salt concentration.
Samples incubated with *B. wiedmanni/cereus*, no salt gave the largest difference, as shown by a high standard error.

Two ways ANOVA with organic acids and carbohydrates concentrations as dependent variables and isolate, autoclaving, salt concentration and time as independent variables showed that isolate was significant only for pyruvic acid (P-value < 0.05), autoclaving and time were significant for all the dependent variables and salt was significant only for lactic acid.
The interactions isolate x autoclaving and isolate x salt were no significant for any of the dependent variables, while isolate x time was significant only for lactic acid.

3.9. Amino acids concentrations detected with High Pressure Liquid Chromatography (HPLC) in cheese and cheese model samples.

High pressure liquid chromathography (HPLC) showed that leucine, lysine and glutamic acids were the most abundant amino acids in all the samples, with concentrations > 1.5 micromole/gr. Histidine, alanine and menthionine showed the lowest concentrations. GABA was not detected in any of the samples.

All amino acids, except for glutamine and citrulline, increased in concentration with sterilization.
With sterilization, L-Glutamine concentrations dropped drastically from values ~ 1 micromole/gr to values ~ 0.

Tabel 4 shows which of the amino acids increased in concentration in the cheese model samples inoculated with *B. wiedmanni/cereus* 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138 during incubation (from Time 0 to Time1).

Tabel 4. In cheese model samples inoculated with *B. wiedmanni/cereus* 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138, increaseed, decreased and conserved amino acids concentrations between Time 0 and Time 1.

Isolate	Increased	Decreased	Conserved
***B. wiedmanni/cereus* 31**	arginine	aspartic acid, asparagine, serine, glycine, threonine citrulline, alanine,valine, menthionine, isoleucine, phenylalanine, tryptophan, leucine, ornithine, lysine	glutamic acid, histidine, tyrosine
***C. sporogenes* 54**	-	aspartic acid, asparagine, serine, glycine, threonine, citrulline, alanine, menthionine, isoleucine, phenylalaline, tryptophan, leucine, ornithine, lysine	glutamic acid, histidine
***C. tyrobutyricum* 138**	asparagine, serine, glycine, threonine citrulline, arginine,alanine, tyrosine, valine, menthionine, isoleucine, phenylalaline, leucine ornithine, lysine	tryptophan	aspartic acid, glutamic acid, histidine

During incubation most amino acids decreased in concentration in samples inoculated with *B. wiedmanni/cereus* 31 and with *C. sporogenes* 54, while in samples inoculated with *C. tyrobutyricum*, the majority of amino acids increased in concentration.

Tabel 5 shows which of the amino acids, in the cheese model samples inoculated with *B. wiedmanni/cereus* 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138, showed highest concentrations at 0% salt and which at 2% salt after incubation.

Tabel 5. In cheese model samples inoculated with *B. wiedmanni/cereus* 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138, amino acids levels at 0% salt concentration and 2% salt concentration.

Isolate	Histen concentration at 0% salt	Highest concentration at 2% salt
***B. wiedmanni/cereus* 31**	aspartic acid, asparagine, serine, glycine, threonine, citrulline, arginine, alanine, tyrosine, valine	tryptophan
***C. sporogenes* 54**	aspartic acid	serine, glycine, threonine, citrulline, arginine, alanine, tyrosine, valine, isoleucine, phenylamine, tryptophan, leucine lysine, ornithine
***C. tyrobutyricum* 138**	glutamic acid, asparagine, glycine threonine, citrulline, arginine	serine, alanine, tyrosine, valine, methionine, isoleucine, phenylamine, leucine, lysine, ornithine

After incubation, in samples inoculated with *B. wiedmanni/cereus* 31, most amino acids showed highest concentrations at 0% salt, while in samples inoculated with clostridia most amino acids showed highest concentrations at 2% salt.

At Time 1, most amino acids (aspartic acid, glutamic acid, asparagine, histidine, glycine threonine, citrulline, arginine, alanine, tyrosine, valine, methionine, isoleucine, phenylamine,

leucine, lysine and ornithine) showed highest concentrations for both the salt levels in samples inoculated with *C. tyrobutyricum*.

Two ways ANOVA with amino acid concentrations as dependent variables and isolate, autoclaving, salt concentration and time as independent variables, showed that isolate was significant for pyruvic acid serine, glycine, alanine, methionine and lysine (P-value < 0.05), autoclaving was significant for all the amino acids except for glutamic acid, glycine and citrulline and time was significant for serine, glycine, threonine, citrulline, arginine, alanine, valine, methionine, isoleucine, tryptophan and leucine and salt was significant only for lactic acid. Salt gave P-value < 0.05 only for aspartic acid, glutamic acid and isoleucine.
The interactions Isolate x Autoclave gave P-value > 0.05 in all the models. Isolate x Time gave P-value < 0.05 only for citrulline. Isolate x Salt gave P-value < 0.05 only for serine.

.

.

4. Discussion.

4.1. Growth and proteolytic activity of sporeformers in UHT milk.

In the following chapters, the results obtained for bacterial growth and casein degradation patterns in UHT milk are going to be discussed in relation to the findings about metabolic behavior of the species available in the literature.

4.1.1. Bacterial growth in UHT milk.

All the isolates were able to grow in UHT milk, as indicated by the he differences between cell concentrations determined after 24 hours and after four days incubation.

The strong heat treatment to which the UHT milk is subjected, typically 130º-140 ºC for 3-5 seconds (Fox et al., 1998) eliminates all bacterial competitors and inactivates inhibitors indigenous to the milk, for example immunoglobulins and the lactoperoxidase system.

In addition, at temperatures above 100 ºC, lactose is partially degraded to acids, where formic acid is the most abundant, which may have a stimulating effect on several bacterial strains (Walstra, 2014).

Growth of *Bacillus* ssp. observed in UHT milk was in line with the literature, where contaminations of processed milk by Bacillus cereus group members are abundantly reported (Bartoszewicz & Marjańska, 2017).

While *Clostridium* spp. usually do not grow in milk as it is too aerobic (Walstra, 2014), the relatively extensive clostridial growth observed in UHT milk may be explained by the anaerobic conditions in which the samples were incubated. Clostridial growth may have also been stimulated by the minor content of acids that may be found in UHT milk. In addition, the temperature used for incubation of the samples, 37 ºC, was chosen to guarantee optimal growth of mesophilic bacteria, to which category *Clostridium* ssp. belong (Schiraldi & De Rosa, 2016).

However, it is important to remind that the cell counting performed with the Bürker counting chamber did not allow to distinguish between viable and dead cells and that the results given in cells/mL represent the overall cell concentration only.

Cell concentration by itself cannot therefore represent bacterial growth with accuracy, but it can be used as an indicator.

In order to have an account of the concentration of viable cells in the samples, cell counting should have been performed under phase contrast microscopy, or samples should have been

prepared with dyes apt to detection of dead cells, such as Trypan blue (Fang & Trewyn, 2012). Alternatively, bacterial growth could have been determined in colony forming units per mL (CFU/mL) by means of plate counting. This was the method originally chosen for determination of bacterial growth but, as it requires incubation, it was not used due to the reduced accessibility to the laboratories during the spreading of Covid-19.

4.1.2. Casein degradation tests and proteolytic patterns in UHT milk.

The results of the casein degradation on skim milk agar were expected for *Bacillus* ssp. The high proteolytic activity of *Bacillus* spp. on casein has been amply demonstrated (Janštová et al., 2006). The diameter of the clear zones gives an indication of the protein degradation ability, which is defined in the literature as strong and weak, although the parameters for the definition has not been found. In some cases, the extension of casein degradation observed on skim milk agar was not confirmed by the azocasein assay, as strains that gave a relatively weak casein degradation on skim milk agar showed absorbance values higher than those given by strains that on skim milk agar formed relatively large clear zones.

The results obtained on skim milk agar where more in line with the CE results for samples incubated for four days, as the extension of the clear zones found correspondence in the degrees of casein degradation shown by the plots.

It has been previously demonstrated that the degree of milk protein degradation by means of bacterial proteases is protein fraction specific and that K and β-casein have higher protease sensitivity than of α caseins, probably due to the difference in the amino acids side chains between these caseins (Choudhery & Mikolajcik, 1970).

A freshly published study on *B. cereus* protease activity in UHT milk reported, for an incubation temperature of 28°C, complete degradation of K casein already after 12 hours and a high degree of degradation of β and α_s caseins after 20 hours of incubation (Yang et al., 2021). The CE plot obtained for investigated *Bacillus* ssp. after 24 hours of incubation was in line with these findings.

The CE plots for samples incubated for four days showed an increased degree of casein degradation, which, as suggested by cell concentrations, may be also a consequence of growth. The differences in the degree of hydrolysis between *Bacillus* isolates after four days of incubation may be explained by the wide variety of proteases produced by this species, of witch quantity and ratio are strain dependent (Němečková et al., 2009).

Putting the azocasein assay results in relation with the CE plots and with the literature, absorbance values given by the azocasein assay for the investigated *Bacillus* ssp. were expected to be generally higher.
In relation to samples incubated for 24 hours, while the CE plots for the different *Bacillus* ssp. were very similar and it was possible to report them with a representative, absorbance values for the same *Bacillus* ssp. were relatively heterogeneous.
Incongruencies between the CE plots and azocasein assay results were also seen for samples incubated for four days. Whereas the CE plots showed increased protein degradation with increased incubation time, the absorbance values were lower after four days of incubation than after 24 hours for all the isolates, which is illogical.

As for the clostridial isolates, the inability of zone formation showed by *C. tyrobutyricum* and *C. bejerinchii/diolis* in skim milk agar may be explained by the saccharolytic metabolism of these species.
C. tyrobutyricum has its favorite carbon source in glucose. Alternatively, in addition to pyruvic acid and lactic acid, it can ferment xylose (Linger et al., 2020) but, in contrast with other saccharolytic clostridia, it is not able of lactose fermentation (Ivy & Wiedmann, 2014).
C. diolis and *C. bejerinckii* , which have been very recently reclassified as synonyms, are able to ferment several carbohydrates, in addition to glycerol (Sedlar et al., 2021).
Several studies concluded that both *C. tyrobutyricum* and *C.diolis* do not require amino acids to grow (Storari et al., 2016), which may suggest a very limited or no proteases production. The results seen on skim milk agar can be therefore justified. The ability of zone formation showed by *C. sporogenes* can, on the other hand, be explained by the fact that although this species is able of sugar fermentation, its growth relies mainly on amino acids availability (Flythe & Russell, 2005) and it can actually grow on amino acids only (Poehlein et al., 2015).
The casein degradation test by growth on skim agar gave unexpected results for *C. tyrobutyricum* 19, which actually showed clear zones and higher casein degradation ability than several *Bacillus* ssp. As this result is not in line with the literature and was not supported neither by CE plots nor by the azocasein assay, it may be possible to attribute it to mere plate contamination. The fact that the isolate was the only *C. tyrobutyricum* strain to be able to grow in skim milk agar supports this conclusion.

The poor growth showed by isolates of *C. tyrobutyricum* and *C. bejerinchii/diolis* on skim milk agar may be to ascribe to the fact that these *Clostridium* spp. do not produce β- galactoctisade and thus they could not utilize the lactose present in the medium (Abdul Khalil et al., 2014) .

The results from the casein degradation test by growth on skim milk agar for *C. sporogenes* were only partially confirmed by the CE plots. Whereas on skim milk agar *C. sporogenes* showed a strong proteolytic activity which was even higher than that of most bacilli, the CE plots showed some degree of proteolysis, but the degradation was lower than that of *Bacillus* ssp.

As for *C. tyrobutyricum* and *C. diolis/beijerinckii*, the results were consistent with what could be expected from the literature: in all assays low or no casein degradation were observed (even after four days of incubation).

The azocasein assay results for clostridia were more in line with the literature and with the CE plots than those for *Bacillus* spp., as very low absorbance values were seen for *C. tyrobutyricum* and *C. diolis/beijerinckii*, while higher absorbance values were seen for C. *sporogenenes* (although for only one isolate out of two).

The results for S. epidermidis obtained on skim milk agar were confirmed by the azocasein assay, as very low absorbance was measured for the isolate which did not showed any zone formation. The macroscopic difference in proteolytic ability between the two strains was confirmed once more by the CE plots. S. epidermidis is a commensal opportunist pathogen found on the skin and mucosa of mammals. Its presence in milk has been associated with bovine subclinical mastitis (Thorberg et al., 2009). Although the proteases produced by this staphylococcus are limited in number, they have been found to play an important role in biofilm formation and for virulence expression (Martínez-García et al., 2018). When testing the Esp protease activity of *S. epidermis* with the azocasein assay, absorbance values above 0.3 OD440 nm has been reported (Chen et al., 2013). However, Esp production is strain dependent, and S. epidermidis is categorized both as Esp^+ and Esp^- (Iwase et al., 2010). This strain dependent proteinase production could explain the macroscopical differences in casein degradation ability showed by the two staphyloccal strains on skim milk agar, in the azocasein assay and in CE plots.

Comparing the different methods for determination of protein degradation, spectrofometric methods such as azocasein have the advantage to be quick, but they are regarded as less sensitive than methods based on direct analysis of casein degradation such as CE (Němečková et al., 2009), (BUTTON et al., 2011)This could maybe explain that incongruencies found for the azocasein assay results.

4.2. Growth and metabolism of *B. wiedmanni/cereus*, *C. sporogenes* and *C. tyrobutyricum* in the cheese model.

In the following chapters, the results obtained for growth and metabolism pathways in the cheese model are going to be put in relation to the findings described in chapter 4.1. and those available in the literature.

4.2.1. Carbohydrates, organic acids and amino acids profiles in cheese previous and after autoclaving.

The absence of residual lactose, glucose and citric acid in the cheese was in line with the data given by the literature for fresh cheese (Cebeci et al., 2020) and is to ascribe to the lactose and citrate metabolism of LAB (Walstra, 2014).

The lack of galactose observed in the cheese is probably to ascribe to a mistake in the handling of the results. Galactose can be fermented by LAB either through tagatose 6-P pathway, Leloir pathway or a combination of the two. However, the presence of these pathways in the metabolic machinery of LAB is strain dependent and residual galactose is usually found in fresh cheese (Cebeci et al., 2020), (Iskandar et al., 2019).

The cheese had, as expected, high levels of lactic acids as, this organic acid is the primary end product of the lactate metabolism (Walstra, 2014).

The decrease of lactic and acetic acids concentrations due to autoclaving may be to ascribe to the high chemical reactivity of these acids which make them object of thermal degradation by means of decarboxylation and oxidation if high temperatures are applied (Komesu et al., 2017) and (Li et al., 2017). The increase of pyruvic acid levels with sterilization can be looked upon at as a consequence of acetic and lactic acid degradation (Kamel & Halperin, 2017).

The extensive increase of DL- pyroglutamic acid with sterilization was most probably due to degradation of the amino acid glutamine, which is converted first to glutamic acid and then to DL- pyroglutamic acid with increasing temperature (Airaudo et al., 2006). This is confirmed

by the dramatic drop of glutamine levels observed after sterilization of the cheese bags and may also explain why the glutamic acid concentration was slightly higher in the cheese model than in the cheese.

Sterilization at 121 ºC for 20 minutes led, as expected, to casein degradation and consequent liberation of free amino acids (Walstra, 2014). The increase in concentration of most free amino acids except glutamine and citrulline observed with sterilization contrasts with the availability of amino acids generally reported for other sterilized dairy products. A study conducted on infant formula reported an average loss of free amino acids of 22, 6% for the sterilized product, where valine, citrulline and glutamine being decreased with over 61% (Yeung et al., 2006). Here, however, amino acids were lost as they got involved in Maillard reaction together with lactose. As lactose was absent in the cheese used for the cheese model, The increased amino acid concentrations observed in the cheese model after autoclaving may be therefore explained by the absence of lactose in the cheese and by the fact that no Maillard reaction took place.

4.2.2. Growth and metabolism *of B. wiedmanni/cereus* in the cheese model.

The relatively extensive growth showed by *B. wiedmanni/cereus* in the cheese model was in contrast with findings relative to other daily products (Tirloni et al., 2020a). The inhibitory effects of LAB on *B. cereus* has been demonstrated, as well as in raw milk, in yogurt, in fresh cheese, in gouda and taleggio (Tirloni et al., 2020a), this suggesting that the lack of competitors was determinant for growth in the cheese model. The role of LAB as biopreservatives in cheese is currently an object of research, and efforts have been made in order to identify strains able to combine the preservative effect together with a minimal impact on the sensory profile of the product (Tirloni et al., 2020a).

The fact that growth of *B. wiedmanni/cereus* was not inhibited by the high lactic acid concentrations found in the cheese model is in line with the literature and indicates that the inhibitory effect of LAB is not due to acid production only, but it is the result of a synergy of cofactors, where nutrient depletion and production of antimicrobial compounds play an important role (Rukure & Bester, 2001).

Surprisingly, salt concentration was not a significant factor for *B. wiedmanni/cereus* growth. In vitro studies showed that, although some *B. cereus* strains are highly salt tolerant, increased salt concentration and growth of vegetative cells are negatively correlated (Raevuori & Genigeorgis, 1975). A study conducted on Taleggio cheese indicates salting as a cofactor for

B. cereus inhibition (Tirloni et al., 2020b). For these reasons a less extended growth could have been expected at 2% salt concentration.

As concentrations of galactose, pyruvic acid and of most amino acids decreased during incubation, it may be possible to conclude that *B. wiedmanni/cereus* 31 utilized all these compounds as energy sources. Although the ability to ferment galactose is strain dependent (Warda et al., 2016) the probability to see it for this *B. wiedmanni/cereus* 31 was quite high, as this strain was isolated in the dairy environment. The utilization of pyruvic acid as energy source was also expected, as pyruvic acid is an intermediate product in the *B. cereus* fermentation pathway (Rosenfeld et al., 2005). On the other hand, the fact that lactic and acetic acids concentrations decreased during incubation was somehow unexpected, as these acids are end products of the fermentative pathway of *B. cereus* (Rosenfeld et al., 2005).

When in acid conditions, *B. cereus* can regulate internal pH by means of three different pathways that involve the utilization of amino acids: the arginine deaminase (DA) pathway, the glutamate decarboxylase system and the lysine decarboxylase system (Duport et al., 2016). The DA, which has ornithine, ammonium and CO_2 as main end products, is also used in anaerobic conditions to provide energy (Novák et al., 2016). The decrease of glutamic acid and of lysine concentrations observed after incubation may indicate that the isolate used one or both decarboxylase systems as a defense against acid shock. On the other hand, deamination of arginine did not take place, as the levels of arginine increased and ornithine decreased during incubation.

Salt addition resulted to be somehow stimulating for the metabolism of *B. wiedmanni/cereus* 31. This was seen in relation to both the proteolytic activity and the utilization of galactose, organic acids and the majority of amino acids. A possible explanation is that higher amounts of nutrients are required by *B. wiedmanni/cereus* for response to salt stress and adaptation to mild salinity conditions.

4.2.3. Growth and metabolism *of C.sporogenes* in the cheese model.

As *C. sporogenes* is commonly isolated in different cheese productions and it is the most abundant bacterial species in processed cheese and Manchego (Brändle et al., 2016), the extensive growth of *C. sporogenes* 54 in the cheese model did not cause surprise.

Like for *B. wiedmanni/cereus, C. sporogenes* was also expected to show a reduced growth at higher salt concentration. In this sense the results obtained were not consistent with others available in the literature. A study conducted on canned butter reported salt concentration as a significant factor for *C. sporogenes* growth. Moreover, a salt concentration above 1,6 % corresponded to growth decrease. However, the same study pointed out the ability of some strains to growth at salt concentrations above 2%, this suggesting a strain dependent salt tolerance (Taylor et al., 2013). Nevertheless, adaptability of *C. sporogenes* to acidic and saline environments is amply demonstrated by the literature. Spores of this species are able to germinate even at 4% salt concentration, if at pH range > 5.00 (Valero et al., 2020). The growth of C. sporongens observed at 2% salt concentration may be due to the mild pH of the cheese used in the trial was (> 5.00).

As previously explained, *C. sporogenes* is able of carbohydrate fermentation (Flythe & Russell, 2005). This species is able of pyruvic acid and lactic acid fermentation, although the favorite metabolic pathway is usually that of amino acid degradation through Strickland reactions (Poehlein et al., 2015). Several amino acids have been reported to be essential for the growth of *C. sporogenes*, although researchers have not yet agreed on a definitive list, this due to the great heterogeneity of nutritional needs showed by the different strains (Storari et al., 2016).
The levels of galactose and pyruvic acids observed in the cheese model after incubation are in line with the literature (Flythe & Russell, 2005), as well as the degradation patterns seen for most of the amino acids.

Surprisingly, lactic acid concentration increased during incubation, although lactic acid should have been used, together with pyruvic acid, as fermentation starting point. In addition, neither the lactic acid fermentative pathways nor the amino acid degradation by means of Strickland reactions justify a lactic acid production.
The Strickland reaction pathway has acetic acid as end product (Piveteau, 2017). As external acetic acid is converted into butyric acid in the fermentation pathway (Detman et al., 2019), the fact that the acetic acid concentration increased during incubation may identify the amino acids as the favorite energy source of *C. sporogenes* 54 when easily available carbohydrates are missing, this being in line with the literature (Flythe & Russell, 2005). As a confirmation, among the amino acids most utilized by C. sporogenes there are asparagine, glycine and menthionine, which usually can form Strickland pairs (de Vladar, 2012).

In contrast to *B. wiedmanni/cereus*, *C. sporogenes* showed at 2 % salt concentration a somehow reduced metabolic activity in terms of amino acid degradation, galactose and pyruvic acid consumption and acetic acid production, although salt was not a significant factor for any of these dependent variables. The proteolytic activity pattern given by the CE plots are openly in contrast with these findings.

4.2.3. Growth and metabolism *of C. tyrobutyricum* in the cheese model.

Several studies have pointed out how *C. tyrobutyricum* resistance to acidity and salinity, as well as temperature sensitivity, are highly strain dependent and therefore very heterogenic (Podrzaj et al., 2020). Interestingly, a recent study has demonstrated that cheese vegetative cells from spores germinated under stress conditions are less sensitive to acid and salts than vegetative cells that occur with recontamination (Silvetti et al., 2018).

A study conducted on salt tolerance of this species revealed that of the ten *C. tyrobutyricum* strains tested all were able to grow at 2% salt concentration, and that of these, 5 were able to grow even at a salt concentration of 3% (Ruusunen et al., 2012). All this considering, it was not surprising that salt concentration was not a significant factor for growth of *C. tyrobutyricum* 138 in the cheese model, nor that a somehow higher growth was achieved at 2 % salt.

Surprisingly, *C. tyrobutyricum* 138 was most probably able to ferment galactose, as indicated by the decreased galactose concentration after incubation, whereas no mention of galactose fermentation ability have been found in the literature.
Like *C. sporogenes, C. tyrobutyricum* in cheese ferments pyruvic and lactic acids through the butyric acid pathway. The levels of pyruvic acid observed in the cheese model after incubation were in line with the literature. However, like for *C. sporogenes*, the final lactic acid concentrations were not congruent with the lactic acid fermentation pathway. In principle, also acetic acid should have decreased in concentration after incubation, thus showing a positive correlation with lactic acid levels. About the consumption of acetic acid by *C. tyrobutyricum,* references with opposite meaning are given by the literature. Some studies report lower acetic acid concentrations for cheese contaminated with by *C. tyrobutyricum* than for control samples, while others suggest an acetic acid production under fermentation (Podrzaj et al., 2020).

With the only exception of tryptophan and isoleucine, no amino acid degradation was seen for *C. tyrobutyricum* 138. These findings were consistent with the literature, which describes the species as saccharolytic and butyric, while regards amino acid as non-essential for growth. Genes encoding for biosynthesis of all the amino acids were in fact recently identified for *C. tyrobutyricum* (Storari et al., 2016).

4.3. Conclusions.

All the isolate showed growth in UHT milk. This media provided high availability of fermentable carbohydrates and no competitive flora. Clostridia could grow in this media, usually unsuitable for their metabolism, most probably because of the favorable temperature and anoxia conditions provided during the incubation of the samples, in combination with the availability of organic acids that could have act as stimulants.

Bacillus ssp. showed extended casein degradation activity in both skim milk agar and UHT milk, where differences between the isolates may be explained by the great variety of proteases produced by the *Bacillus* strains.
Incongruencies were found between the casein degradation obtained on skim milk agar and the CE plots on one side and the results from the azocasein assay on the other, which in most cases gave for *Bacillus* ssp. unexpected low absorbance.

As for the clostridia tested, only *C. sporogenes* showed proteolytic activity. Amino acids are essential nutrients for this species, thus proteases production is required for growth. The proteolytic activity showed by *C. sporogenes* was anyway lower than that given by *Bacillus* ssp.
C. diolis and C. *tyrobutyricum,* on the other hand, showed in UHT milk low or no proteolytic activity at all. These two *Bacillus* ssp. are in fact mainly saccharolytic and do not require amino acids to grow.

All the three strains tested were able to grow in the cheese model with no significant difference in terms of cell concentration, whereas growth of *Bacillus* ssp. in cheese is usually inhibited by LAB.

Autoclaving of the cheese model had a significant effect on organic acids and carbohydrates concentration, providing higher concentrations of DL-pyruvic and pyruvic acid and lower concentrations of acetic and lactic acids than those found in the cheese. In addition, autoclaving significantly increased the concentration of most amino acids.

The three bacterial species tested proved to be quite salt tolerant, as none of them showed to be significantly affected by 2% salt concentration in terms of growth. Salt concentration had also a very limited impact on the metabolic pathways investigated, as only lactic acid among the organic acids and only three amino acids out of 20 were significantly affected.

Althoug the three sporeformers showed significant differences in metabolism only in terms of pyruvic acid and of five amino acids out of 20 (serine, glycine, alanine, menthionine and lysisne), *B. wiedmanni/cereus* and *C. sporogenes* used amino acids as energy source in combination with organic acids and galactose. C. *tyrobutyricum*, on the other end, utilized only organic acids and galactose. In this sense, the metabolic patterns seen in the cheese model confirmed the proteolytic behavior showed by the species in UHT milk.

4.4 Further research.

This master thesis provided knowledge on growth and metabolic pathways of three spore formers that can have detrimental impact for cheese quality. However, further research is necessary in order to acquire a more precise picture that can help the cheese industry.

The present master thesis tested growth and metabolic activity of one single strain for each of the bacterial species investigated. Tests performed on multiple strains could help in determining which metabolic traits are conserved within the species and which instead are strain dependent, and they could give a statistical proportion of how the various metabolic traits occur. The results could be then implemented with those from metagenomic analysis.

Further research is needed in order to get knowledge on how and in which measure growth and metabolism of spore formers can be impacted by cheese acidity and incubation temperature. In this sense, tests could be performed on cheese models with different pH and using temperatures close to those normally in use in the ripening room and during storage.

As this master thesis suffered of the restrictions imposed by the pandemic situation, changes of growth and metabolism over time were not investigated and more research is needed in this sense. In case of clostridia, a longer trial period may make it possible to put in relation metabolic activity and the late blow defect of the cheese, which can take place in different phases of the cheese ripening (Walstra, 2014).

The cheese model used in this master thesis was subjected to autoclaving, which as described in the discussion chapter altered the levels of both organic acids and amino acids. Further research could take into consideration types of cheese model which can provide the organic acids and amino acids concentrations typically found in cheese.

Object of investigation of this master thesis, vegetative cells may differ in stress tolerance and metabolic behaviors from cells originated from spore germination *in situ*. It could be therefore of interest to compare the results obtained withing the present work with those from tests performed with spores.

.

5. Appendix.

A.1. Cell concentrations in inoculated UHT milk samples. For each isolate, cell concentrations in sample set A after 24 hours and four days incubation and in sample set B after four days incubation are given in log cells/mL.

Isolate	Set A, 24 hours incubation (log cells/mL)	Set A, 4 days incubation (log cells/mL)	Set B, 4 days incubation (log cells/mL)
***Staphylococcus epidermidis* 137**	**-**	**-**	8.82
Staphylococcus epidermidis* 137	7.34	8.18	8.50
***Bacillus thuringiensis/cereus/tropicus/wiedmanni* 22**	7.36	8.15	8.64
Bacillus thuringiensis/toyonensis/cereus/paramycoides* 22	7.19	8.12	8.70
***Bacillus wiedmanni/cereus* 31**	7,36	8.20	8.58
Bacillus cereus* 31	6.98	8.08	8.48
***Bacillus proteolyticus/cereus* 55**	7.11	8.72	8.31
***Bacillus cereus* 56**	-	-	8.82
***Clostridium diolis/ beijerinckii* 17**	7.33	8.27	8.41
***Clostridium diolis/beijerinckii* 23**	7.60	8.35	8.54
***Clostridium tyrobutyricum* 19**	7.77	8.39	8.53
***Clostridium tyrobutyricum* 24**	7.32	8.54	8.93
***Clostridium tyrobutyricum* 16**	7.24	8.11	8.26
***Clostridium tyrobutyricum* 138**	7.33	8.33	8.31
***Clostridium tyrobutyricum* 139**	7.33	8.19	8.38
***Clostridium sporogenes* 53**	7.06	8.67	8.43
***Clostridium sporogenes* 54**	7.65	8.36	8.90

For *B. cereus* 56 and isolate *S. epidermidis* 137 cell concentration is given for set B only, as these isolates were not used for inoculation of set A.

A.2. Average absorbance values after 24 hours incubation and after four days incubation at 37°C and Azocasein assay. For distilled water, caseinate and the inoculated samples, average absorbance is given in OD 440 nm.

Sample	Absorbance 24 hours incubation (OD 440 nm)	Absorbance four days incubation (OD 440 nm)
Staphylococcus epidermidis 137	-	0.007
Staphylococcus epidermidis 137*	0.041	0.182
Bacillus thuringiensis /cereus/tropicus/wiedemanni 22	0.102	0.023
Bacillus thuringiensis/toyonensis/cereus/paramycoides 22*	0.048	0.019
Bacillus wiedmanni/cereus 31	0.055	0.044
Bacillus cereus 31*	0.047	0.024
Bacillus proteolyticus/cereus 55	0.022	0.017
Bacillus cereus 56	-	0.028
Clostridium diolis/ beijerinckii 17	0.010	0.018
Clostridium diolis/ beijerinckii 23	0.020	0,017
Clostridium tyrobutyricum 19	0.006	0.014
Clostridium tyrobutyricum 24	0.016	0.013
Clostridium tyrobutyricum 16	0.015	0.019
Clostridium tyrobutyricum 138	0.015	0.016
Clostridium tyrobutyricum 139	0.026	0.016
Clostridium sporogenes 53	0.049	0.038
Clostridium sporogenes 54	0.018	0.020
Distilled water	0.014	0.003
Proteinase K	1.341	1.480

A.3. Cell concentration means in cheese model. The average cells concentration of *Bacillus wiedmanni/cereus* 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138 determined in the cheese model samples at Time 0 and at Time 1 is given in cells/ml.

Mean	***Bacillus wiedmanni/cereus* 31 (cells/ml)**	***Clostridium sporogenes* 54 (cells/ml)**	***Clostridium tyrobutyricum* 138 (cells/ml)**
Tid 0	7.3	7.3	7.3
Tid 1 0% salt	8.3	8.4	8.3
Tid 1 2% salt	8.4	8.3	8.4

The model had a S value of 0.121091 and R-sq = 96.18%.

A.4. P-values for the independent variables bacterium type, salt, time and for their interactions, given cell concentration as dependent variable.

Source	P-value
Isolate	0.786
Salt	0.458
Time	<0.001
Bacterium x Salt	0.570
Bacterium x Time	0.997

A.5. S and R-sq values of the statistical models and P- values of the independent variables salt and time, given growth of *Bacillus wiedmanni/cereus*, *Clostridium sporogenes* and *Clostridium tyrobutyricum* as dependent variables.

Isolate	S	R-square (%)	P value Salt	P-value Time
***Bacillus wiedmanni/cereus* 31**	0.1	97.80	0.433	<0.001
***Clostridium sporogenes* 54**	0.2	93.20	0.766	<0.001
***Clostridium tyrobutyricum* 138**	0.1	97.30	0.255	<0.001

The model for statistical analysis with growth of *Bacillus wiedmanni/cereus* 31 as dependent variable gave ahe highest R-square value (97.80%) and lowest S value (0.092316), while the model for growth of *Clostridium sporogenes* 54 gave lowest R-square value (93.20%) and highest S value (0.157056).

A.6. Pyruvic acid concentration means in cheese and in the cheese model.

Mean values for concentration of pyruvic acid are given in mmol/kg for not sterilized cheese samples (Cheese), cheese model at inoculation time (Time 0) and for cheese model samples inoculated with *Bacillus wiedmanni/cereus* 31 , *C. tyrobutyricum* 138 and *C. sporogenes* 54 with and without salt addition after one week of incubation at 37°C (Time 1).

Mean	*B. wiedmanni/cereus* 31	*C. sporogenes* 54	*C. tyrobutyricum* 138
Cheese (mmol/Kg)	1.74	1.74	1.74
Time 0 (mmol/Kg)	2.82	2.82	2.82
Time 1 (mmol/Kg)	1.72	1.83	1.96
Time 1, 2 % salt (mmol/Kg)	1.62	2.14	2.24

A.7. Standard error (SE) for pyruvic acid concentrations. Standard error (SE) is given for pyruvic acid concentrations determinated at Time 0 and at Time 1 in the cheese model triplicates inoculated with *Bacillus wiedmanni/cereus* 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138.

SE	*B. wiedmanni/cereus* 31	*C. sporogenes* 54	*C. tyrobutyricum* 138
No autoclave	0.02	0.02	0.02
Time 0	0.19	0.96	0.96
Time 1, 0% salt	0.11	0.12	0.13
Time 1, 2 % salt	0.04	0.07	0.02

A.8. Galactose concentration means in cheese and in the cheese model.

Mean values for concentration of galactose are given in mmol/kg for not sterilized cheese samples (Cheese), cheese model at inoculation time (Time 0) and for cheese model samples inoculated with *Bacillus wiedmanni/cereus* 31 , *C. tyrobutyricum* 138 and *C. sporogenes* 54 with and without salt addition after one week of incubation at 37°C (Time 1).

Mean	*B. wiedmanni/cereus* 31	*C. sporogenes* 54	*C. tyrobutyricum* 138
Cheese (mmol/Kg)	0	0	0
Time 0 (mmol/Kg)	0.57	0.57	0.57
Time 1 (mmol/Kg)	0.29	0.34	0.35
Time 1, 2 % salt (mmol/Kg)	0.26	0.42	0.43

A.9.. Standard error (SE) for galactose concentrations. Standard error (SE) is given for galactose concentrations determinated at Time 0 and at Time 1 in the cheese model triplicates inoculated with *Bacillus wiedmanni/cereus* 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138.

SE	*B. wiedmanni/cereus* 31	*C. sporogenes* 54	*C. tyrobutyricum* 138
No autoclave	0	0	0
Time 0	0.07	0.07	0.07
Time 1, 0% salt	0.03	0.03	0.03
Time 1, 2 % salt	0.03	0.02	0.00

A.10. Lactic acid concentration means in cheese and in the cheese model.

Mean values for concentration of lactic acid are given in mmol/kg for not sterilized cheese samples (Cheese), cheese model at inoculation time (Time 0) and for cheese model samples inoculated with *Bacillus wiedmanni/cereus* 31 , *C. tyrobutyricum* 138 and *C. sporogenes* 54 with and without salt addition after one week of incubation at 37°C (Time 1).

Mean	***B. wiedmanni/cereus* 31**	***C. sporogenes* 54**	***C. tyrobutyricum* 138**
Cheese (mmol/Kg)	87.1	87.1	87.1
Time 0 (mmol/Kg)	76.3	76.3	76.3
Time 1 (mmol/Kg)	74.2	83.5	83.6
Time 1, 2 % salt (mmol/Kg)	70.1	81.4	76.9

7.11. Standard error (SE) for lactic acid concentrations. Standard error (SE) is given for lactic acid concentrations determinated at Time 0 and at Time 1 in the cheese model triplicates inoculated with *Bacillus wiedmanni/cereus* 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138.

SE	*B. wiedmanni/cereus* 31	*C. sporogenes* 54	*C. tyrobutyricum* 138
No autoclave	0.17	0.76	0.17
Time 0	2.28	2.28	2.28
Time 1, 0% salt	5.75	2.19	2.49
Time 1, 2 % salt	3.03	2.13	1.01

A.12. Acetic acid concentration means in cheese and in the cheese model.

Mean values for concentration of acetic acid are given in mmol/kg for not sterilized cheese samples (Cheese), cheese model at inoculation time (Time 0) and for cheese model samples

inoculated with *Bacillus wiedmanni/cereus* 31 , *C. tyrobutyricum* 138 and *C. sporogenes* 54 with and without salt addition after one week of incubation at 37°C (Time 1).

Mean	***B. wiedmanni/cereus* 31**	***C. sporogenes* 54**	***C. tyrobutyricum* 138**
Cheese (mmol/Kg)	7.95	7.95	7.95
Time 0 (mmol/Kg)	7,.3	7.23	7.23
Time 1 (mmol/Kg)	7.93	8.99	9.58
Time 1, 2 % salt (mmol/Kg)	7.47	8.99	8.81

A.13. Standard error (SE) for acetic acid concentrations. Standard error (SE) is given for acetic acid concentrations determinated at Time 0 and at Time 1 in the cheese model triplicates inoculated with *Bacillus wiedmanni/cereus* 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138.

SE	*B. wiedmanni/cereus* 31	*C. sporogenes* 54	*C. tyrobutyricum* 138
No autoclave	0.06	0.06	0.06
Time 0	0.17	0.17	0.17
Time 1, 0% salt	0.85	0.20	0.15
Time 1, 2 % salt	0.21	0,37	0.08

A.14. D acidL-pyroglutamic concentration means in cheese and in the cheese model.

Mean values for concentration of DL-pyruoglutamic acid are given in mmol/kg for not sterilized cheese samples (Cheese), cheese model at inoculation time (Time 0) and for cheese model samples inoculated with *Bacillus wiedmanni/cereus* 31 , *C. tyrobutyricum* 138 and *C. sporogenes* 54 with and without salt addition after one week of incubation at 37°C (Time 1).

Mean	***B. wiedmanni/cereus* 31**	***C. sporogenes* 54**	***C. tyrobutyricum* 138**
Cheese (mmol/Kg)	0.07	0.07	0.07
Time 0 (mmol/Kg)	1.25	1.25	1.25
Time 1 (mmol/Kg)	1.12	1.2	1.2
Time 1, 2 % salt (mmol/Kg)	1.03	1.23	1.3

A.15. Standard error (SE) for DL-pyroglutamic acid concentrations. Standard error (SE) is given for DL-pyroglutamic acid concentrations determinated at Time 0 and at Time 1 in the cheese model triplicates inoculated with *Bacillus wiedmanni/cereus* 31, *C. sporogenes* 54 and *C. tyrobutyricum* 138.

SE	*B. wiedmanni/cereus* 31	*C. sporogenes* 54	*C. tyrobutyricum* 138
No autoclave	0.01	0.01	0.01
Time 0	0.08	0.08	0.08
Time 1, 0% salt	0.25	0.15	0.50
Time 1, 2 % salt	0.08	0.07	0.02

A.16. S and R-sq values of the statistical models for organic acids and carbohydrates concentration, together with P- values of the independent variables Isolate, Autoclave, Time, Salt and time and the interactions Isolate x Autoclave, Isolate x Time and Isolate x Salt.

Organic acid/ carbohydrate	S	R-square	P-value Isolate	P-value Autoclave	P-value Time	P-value Salt	P-value Isolate x Autoclave	P-value Isolate x Time	P-value Isolate x Salt
Pyruvic acid	17.4669	88.46%	0.028	<0.001	<0.001	0.087	1.000	0.585	0.170
Galactose	12.2642	93.53 %	0.070	<0.001	<0.001	0.161	1.000	0.731	0.273
Lactic acid	389.823	70.75%	0.096	<0.001	0.051	0.046	1.000	0.112	0.654
Acetic acid	31.3262	75.40%	0.060	0.007	<0.001	0.056	1.000	0.036	0.685
DL-pyroglutamic acid	10.8430	98.10 %	0.063	<0.001	0.066	0.726	1.000	0.623	0.332

The model for statistical analysis with DL-pyroglutamic acid as dependent variable gave the highest R-square value (98.10%) and lowest S value (10.8430), while the model for lactic acid gave lowest R-square value (70.75%) and highest S value (389.823).

Table 17. S and R-sq values of the statistical models for amino acids concentration, together with P- values of the independent variables Isolate, Autoclave, Time, Salt and time and the interactions Isolate x Autoclave, Isolate x Time and Isolate x Salt.

Amino acid	S	R-square	P-value Isolate	P-value Autoclave	P-value Time	P-value Salt	P-value Isolate x Autoclave	P-value Isolate x Time	P-value Bacterium x Salt
Aspartic acid	0.0249878	67.63%	0.170	<0.001	0.441	0.029	1.000	0.610	0.253

Glutamic acid	0.0737127	50.19%	0.101	0.691	0.730	0.024	1.000	0.187	0.242
Asparagine	0.0411089	57.64%	0.240	<0.001	0.968	0.328	1.000	0.540	0.373
Serine	0.0276189	61.53%	0.015	0.001	0.027	0.435	1.000	0.492	0.047
Glutamine	0.0209502	99.85%	0.991	<0.001	0.931	0.819	1.000	0.932	0.954
Histidine	0.0154776	49.34%	0.690	<0.001	0.265	0.641	1.000	0.758	0.923
Glycine	0.0259856	61.63%	0.040	0.707	0.028	0.907	1.000	0.047	0.592
Threonine	0.0158605	55.99%	0.051	0.001	0.037	0.374	1.000	0.468	0.216
Citrulline	0.0185023	66.59%	0.055	0.498	0.030	0.960	1.000	0.014	0.272
Arginine	0.0095088	80.49%	0.110	0.003	<0.001	0.265	1.000	0.148	0.158
Alanine	0.0448631	75.83%	0.009	<0.001	0.041	0.678	1.000	0.078	0.133
Tyrosine	0.0445948	92.50%	0.075	<0.001	0.454	0.027	1.000	0.317	0.369
Valine	0.0584359	81.41%	0.081	<0.001	0.016	0.085	1.000	0.271	0.219
Menthionine	0.0153740	86.94%	0.012	<0.001	0.014	0.083	1.000	0.257	0.082
Isoleucine	0.0369624	87.30%	0.083	<0.001	<0.001	0.026	1.000	0.259	0.282
Phenylanine	0.0485006	92.00%	0.110	<0.001	0.433	0.302	1.000	0.169	0.639
Tryptophan	0.0094575	54.06%	0.945	<0.001	0.007	0.085	1.000	0.394	0.255
Leucine	0.111187	86.00%	0.081	<0.001	0.049	0.143	1.000	0.126	0.358
Ornithine	0.0529339	55.48%	0.119	0.001	0.577	0.809	1.000	0.293	0.380
Lysine	0.0850234	69.15%	0.040	<0.001	0.229	0.322	1.000	0.200	0.314

The model for statistical analysis with histidine as dependent variable gave the lowest R-square value (49.34%), while the model for glutamine gave the highest R-square value (99.85). Lowest S value was given by the statistical model for tryptophan (0.0094575), while the highest S value was that for the model of leucine (0.111187).

6. Literature.

Abdul Khalil, K., Mustafa, S., Mohammad, R., Bin Ariff, A., Shaari, Y., Abdul Manap, Y., Ahmad, S. A. & Dahalan, F. A. (2014). Optimization of milk-based medium for efficient cultivation of Bifidobacterium pseudocatenulatum G4 using face-centered central composite-response surface methodology. *BioMed research international*, 2014: 787989-787989. doi: 10.1155/2014/787989.

Airaudo, C., Gayte-Sorbier, A. & Armand, P. (2006). Stability of Glutamine and Pyroglutamic Acid under Model System Conditions: Influence of Physical and Technological Factors. *Journal of Food Science*, 52: 1750-1752. doi: 10.1111/j.1365-2621.1987.tb05926.x.

Andreani, N. A., Carraro, L., Fasolato, L., Balzan, S., Lucchini, R., Novelli, E. & Cardazzo, B. (2016). Characterisation of the Thermostable Protease AprX in Strains of Pseudomonas Fluorescens and Impact on the Shelf-life of Dairy Products: Preliminary Results. *Italian journal of food safety*, 5 (4): 6175-6175. doi: 10.4081/ijfs.2016.6175.

Bartoszewicz, M. & Marjańska, P. S. (2017). Milk-originated Bacillus cereus sensu lato strains harbouring Bacillus anthracis-like plasmids are genetically and phenotypically

diverse. *Food Microbiology*, 67: 23-30. doi: https://doi.org/10.1016/j.fm.2017.05.009.
Brändle, J., Domig, K. J. & Kneifel, W. (2016). Relevance and analysis of butyric acid producing clostridia in milk and cheese. *Food Control*, 67: 96-113. doi: https://doi.org/10.1016/j.foodcont.2016.02.038.
Brändle, J., Fraberger, V., Berta, J., Puglisi, E., Jami, M., Kneifel, W. & Domig, K. J. (2018). Butyric acid producing clostridia in cheese – Towards the completion of knowledge by means of an amalgamate of methodologies. *International Dairy Journal*, 86: 86-95. doi: https://doi.org/10.1016/j.idairyj.2018.07.008.
BUTTON, P. D., ROGINSKI, H., DEETH, H. C. & CRAVEN, H. M. (2011). IMPROVED SHELF LIFE ESTIMATION OF UHT MILK BY PREDICTION OF PROTEOLYSIS. *Journal of Food Quality*, 34 (4): 229-235. doi: https://doi.org/10.1111/j.1745-4557.2011.00394.x.
Cebeci, A., Yaman, M., Yalcin, B. & Gunes, F. (2020). Determination of carbohydrate amounts of various cheese types presented to sale in the market. 2455-4898.
Chen, C., Krishnan, V., Macon, K., Manne, K., Narayana, S. V. L. & Schneewind, O. (2013). Secreted proteases control autolysin-mediated biofilm growth of Staphylococcus aureus. *The Journal of biological chemistry*, 288 (41): 29440-29452. doi: 10.1074/jbc.M113.502039.
Choudhery, A. K. & Mikolajcik, E. M. (1970). Activity of Bacillus cereus Proteinases in Milk1. *Journal of Dairy Science*, 53 (3): 363-366. doi: https://doi.org/10.3168/jds.S0022-0302(70)86210-5.
D'Incecco, P., Pellegrino, L., Hogenboom, J. A., Cocconcelli, P. S. & Bassi, D. (2018). The late blowing defect of hard cheeses: Behaviour of cells and spores of Clostridium tyrobutyricum throughout the cheese manufacturing and ripening. *LWT*, 87: 134-141. doi: https://doi.org/10.1016/j.lwt.2017.08.083.
Dash, S., Ng, C. Y. & Maranas, C. D. (2016). Metabolic modeling of clostridia: current developments and applications. *FEMS Microbiology Letters*, 363 (4). doi: 10.1093/femsle/fnw004.
de Vladar, H. P. (2012). Amino acid fermentation at the origin of the genetic code. *Biology direct*, 7: 6-6. doi: 10.1186/1745-6150-7-6.
Detman, A., Mielecki, D., Chojnacka, A., Salamon, A., Błaszczyk, M. K. & Sikora, A. (2019). Cell factories converting lactate and acetate to butyrate: Clostridium butyricum and microbial communities from dark fermentation bioreactors. *Microbial Cell Factories*, 18 (1): 36. doi: 10.1186/s12934-019-1085-1.
Doyle, C. J., Gleeson, D., Jordan, K., Beresford, T. P., Ross, R. P., Fitzgerald, G. F. & Cotter, P. D. (2015). Anaerobic sporeformers and their significance with respect to milk and dairy products. *International Journal of Food Microbiology*, 197: 77-87. doi: https://doi.org/10.1016/j.ijfoodmicro.2014.12.022.
Duport, C., Jobin, M. & Philippe, S. (2016). Adaptation in Bacillus cereus: From Stress to Disease. *Frontiers in Microbiology*, 7. doi: 10.3389/fmicb.2016.01550.
Ehling-Schulz, M., Lereclus, D. & Koehler, T. M. (2019). The Bacillus cereus Group: Bacillus Species with Pathogenic Potential. *Microbiology spectrum*, 7 (3): 10.1128/microbiolspec.GPP3-0032-2018. doi: 10.1128/microbiolspec.GPP3-0032-2018.
Fang, I. J. & Trewyn, B. G. (2012). Chapter three - Application of Mesoporous Silica Nanoparticles in Intracellular Delivery of Molecules and Proteins. In Düzgüneş, N. (ed.) vol. 508 *Methods in Enzymology*, pp. 41-59: Academic Press.

Ferragut, V. & Trujillo, A.-J. (2008). Semi-Hard Cheese – Cheese Making Technology. In, pp. 155-160.

Flythe, M. D. & Russell, J. B. (2005). The ability of acidic pH, growth inhibitors, and glucose to increase the proton motive force and energy spilling of amino acid-fermenting Clostridium sporogenes MD1 cultures. *Archives of Microbiology*, 183 (4): 236-242. doi: 10.1007/s00203-005-0765-x.

Fox, P. F., Uniacke-Lowe, T., McSweeney, P. L. H. & O'Mahony, J. A. (1998). Dairy Chemistry and Biochemistry. *Dairy Chemistry and Biochemistry*.

Gómez-Torres, N., Garde, S., Peirotén, Á. & Ávila, M. (2015). Impact of Clostridium spp. on cheese characteristics: Microbiology, color, formation of volatile compounds and off-flavors. *Food Control*, 56: 186-194. doi: https://doi.org/10.1016/j.foodcont.2015.03.025.

Guinebretière, M.-H., Thompson, F. L., Sorokin, A., Normand, P., Dawyndt, P., Ehling-Schulz, M., Svensson, B., Sanchis, V., Nguyen-The, C., Heyndrickx, M., et al. (2008). Ecological diversification in the Bacillus cereus Group. *Environmental Microbiology*, 10 (4): 851-865. doi: https://doi.org/10.1111/j.1462-2920.2007.01495.x.

Gunetti, M., Castiglia, S., Rustichelli, D., Mareschi, K., Sanavio, F., Muraro, M., Signorino, E., Castello, L., Ferrero, I. & Fagioli, F. (2012). Validation of analytical methods in GMP: The disposable Fast Read 102® device, an alternative practical approach for cell counting. *Journal of translational medicine*, 10: 112. doi: 10.1186/1479-5876-10-112.

Huang, Y., Flint, S. H. & Palmer, J. S. (2020). Bacillus cereus spores and toxins – The potential role of biofilms. *Food Microbiology*, 90: 103493. doi: https://doi.org/10.1016/j.fm.2020.103493.

Iskandar, C. F., Cailliez-Grimal, C., Borges, F. & Revol-Junelles, A.-M. (2019). Review of lactose and galactose metabolism in Lactic Acid Bacteria dedicated to expert genomic annotation. *Trends in Food Science & Technology*, 88: 121-132. doi: https://doi.org/10.1016/j.tifs.2019.03.020.

Ivy, R. A. & Wiedmann, M. (2014). CLOSTRIDIUM | Clostridium tyrobutyricum. In Batt, C. A. & Tortorello, M. L. (eds) *Encyclopedia of Food Microbiology (Second Edition)*, pp. 468-473. Oxford: Academic Press.

Iwase, T., Uehara, Y., Shinji, H., Tajima, A., Seo, H., Takada, K., Agata, T. & Mizunoe, Y. (2010). Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization. *Nature*, 465 (7296): 346-349. doi: 10.1038/nature09074.

Janštová, B., Dračková, M. & Vorlová, L. (2006). Effect of Bacillus cereus Enzymes on Milk Quality following Ultra High Temperature Processing. *Acta Veterinaria Brno - ACTA VET BRNO*, 75: 601-609. doi: 10.2754/avb200675040601.

Kabelova, I., Dvorakova, M., Cizkova, H., Dostálek, P. & Melzoch, K. (2009). Determination of free amino acids in cheeses from the Czech market. *Czech Journal of Food Sciences*, 27: 143-150. doi: 10.17221/1415-CJFS.

Kable, M. E., Srisengfa, Y., Laird, M., Zaragoza, J., McLeod, J., Heidenreich, J. & Marco, M. L. (2016). The Core and Seasonal Microbiota of Raw Bovine Milk in Tanker Trucks and the Impact of Transfer to a Milk Processing Facility. *mBio*, 7 (4): e00836-16. doi: 10.1128/mBio.00836-16.

Kamel, K. S. & Halperin, M. L. (2017). chapter 6 - Metabolic Acidosis: Acid Gain Types. In Kamel, K. S. & Halperin, M. L. (eds) *Fluid, Electrolyte and Acid-Base Physiology (Fifth Edition)*, pp. 141-170. Philadelphia: Elsevier.

Komesu, A., Martins Martinez, P. F., Lunelli, B. H., Oliveira, J., Wolf Maciel, M. R. & Maciel Filho, R. (2017). Study of Lactic Acid Thermal Behavior Using Thermoanalytical Techniques. *Journal of Chemistry*, 2017: 4149592. doi: 10.1155/2017/4149592.

Kristiansen, H., Skeie, S. & Porcellato, D. (2020). *Kartlegging av sammensetning og overlevelse av sporedannere langs produksjonslinjen for melkepulver med spesielt fokus på Klostridier*. Composition and survival of sporeformers along the milk powder production line with special focus on Clostridia: Norwegian University of Life Sciences, Ås.

Li, Y., Zhou, S., Li, J., yu, m., Chen, K., Wu, Y. & Zhang, Y. (2017). Experimental study of the decomposition of acetic acid under conditions relevant to deep reservoirs. *Applied Geochemistry*, 84. doi: 10.1016/j.apgeochem.2017.07.013.

Linger, J. G., Ford, L. R., Ramnath, K. & Guarnieri, M. T. (2020). Development of Clostridium tyrobutyricum as a Microbial Cell Factory for the Production of Fuel and Chemical Intermediates From Lignocellulosic Feedstocks. *Frontiers in Energy Research*, 8 (183). doi: 10.3389/fenrg.2020.00183.

Martínez-García, S., Rodríguez-Martínez, S., Cancino-Diaz, M. E. & Cancino-Diaz, J. C. (2018). Extracellular proteases of Staphylococcus epidermidis: roles as virulence factors and their participation in biofilm. *Apmis*, 126 (3): 177-185. doi: 10.1111/apm.12805.

Mazzoli, R. (2012). Development of microorganisms for cellulose-biofuel consolidated bioprocessings: Metabolic engineers' tricks. *Computational and structural biotechnology journal*, 3: e201210007. doi: 10.5936/csbj.201210007.

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening. *International Journal of Dairy Technology*, 57 (2-3): 127-144. doi: https://doi.org/10.1111/j.1471-0307.2004.00147.x.

McSweeney, P. L. H., Ottogalli, G. & Fox, P. F. (2017). Chapter 31 - Diversity and Classification of Cheese Varieties: An Overview. In McSweeney, P. L. H., Fox, P. F., Cotter, P. D. & Everett, D. W. (eds) *Cheese (Fourth Edition)*, pp. 781-808. San Diego: Academic Press.

Mehta, D. S., Metzger, L. E., Hassan, A. N., Nelson, B. K. & Patel, H. A. (2019). The ability of spore formers to degrade milk proteins, fat, phospholipids, common stabilizers, and exopolysaccharides. *Journal of Dairy Science*, 102 (12): 10799-10813. doi: https://doi.org/10.3168/jds.2019-16623.

Moe, K. M., Porcellato, D. & Skeie, S. (2013). Metabolism of milk fat globule membrane components by nonstarter lactic acid bacteria isolated from cheese. *J Dairy Sci*, 96 (2): 727-39. doi: 10.3168/jds.2012-5497.

Narvhus, J. A., Østeraas, K., Mutukumira, T. & Abrahamsen, R. K. (1998). Production of fermented milk using a malty compound-producing strain of Lactococcus lactis subsp. lactis biovar. diacetylactis, isolated from Zimbabwean naturally fermented milk. *International Journal of Food Microbiology*, 41 (1): 73-80. doi: https://doi.org/10.1016/S0168-1605(98)00036-1.

Němečková, I., Pechacova, M. & Roubal, P. (2009). Problems with Detection of Proteolytic Microorganisms and Their Undesirable Activities in Milk. *Czech Journal of Food Sciences*, 27: S282-S289. doi: 10.17221/666-CJFS.

Nilsson, K., Abdelghani, A., Burleigh, S., Buhelt Johansen, L., Lindmark-Månsson, H., Paulsson, M. & Glantz, M. (2020). An investigation of the enzymatic cleavage of κ-casein in non-coagulating milk. *International Dairy Journal*, 109: 104754. doi: https://doi.org/10.1016/j.idairyj.2020.104754.

No, I. S. (2004). Cheese and processed cheese–Determination of the total solids content (reference method).
Novák, L., Zubáčová, Z., Karnkowska, A., Kolisko, M., Hroudová, M., Stairs, C. W., Simpson, A. G. B., Keeling, P. J., Roger, A. J., Čepička, I., et al. (2016). Arginine deiminase pathway enzymes: evolutionary history in metamonads and other eukaryotes. *BMC evolutionary biology*, 16 (1): 197-197. doi: 10.1186/s12862-016-0771-4.
Pahlow, G., Muck, R., Driehuis, F., Oude Elferink, S. & Spoelstra, S. F. (2003). Microbiology of Ensiling. In vol. 42, pp. p. 31-93.
Pailin, T., Kang, D. H., Schmidt, K. & Fung, D. Y. C. (2001). Detection of extracellular bound proteinase in EPS-producing lactic acid bacteria cultures on skim milk agar. *Letters in Applied Microbiology*, 33 (1): 45-49. doi: https://doi.org/10.1046/j.1472-765X.2001.00954.x.
Piveteau, S. (2017). *Optimizing hydrolysis and acidogenesis in order to dissolve and recover phosphorus in organic effluents upstream from methane production*.
Podrzaj, L., Burtscher, J., Küller, F. & Domig, K. J. (2020). Strain-Dependent Cheese Spoilage Potential of Clostridium tyrobutyricum. *Microorganisms*, 8 (11). doi: 10.3390/microorganisms8111836.
Poehlein, A., Riegel, K., König, S. M., Leimbach, A., Daniel, R. & Dürre, P. (2015). Genome sequence of Clostridium sporogenes DSM 795T, an amino acid-degrading, nontoxic surrogate of neurotoxin-producing Clostridium botulinum. *Standards in Genomic Sciences*, 10 (1): 40. doi: 10.1186/s40793-015-0016-y.
Porcellato, D., Aspholm, M., Skeie, S. B. & Mellegård, H. (2018). Application of a novel amplicon-based sequencing approach reveals the diversity of the Bacillus cereus group in stored raw and pasteurized milk. doi: https://doi.org/10.1016/j.fm.2018.01.014.
Raevuori, M. & Genigeorgis, C. (1975). Effect of pH and sodium chloride on growth of Bacillus cereus in laboratory media and certain foods. *Applied microbiology*, 29 (1): 68-73. doi: 10.1128/am.29.1.68-73.1975.
Rosenfeld, E., Duport, C., Zigha, A. & Schmitt, P. (2005). Characterization of aerobic and anaerobic vegetative growth of the food-borne pathogen Bacillus cereus F4430/73 strain. *Canadian journal of microbiology*, 51 (2): 149-58.
Ruegg, P. & Reinemann, D. (2002). Milk Quality and Mastitis Tests. *Bov. Pract.*, 36.
Rukure, G. & Bester, B. H. (2001). Survival and growth of Bacillus cereus during Gouda cheese manufacturing. *Food Control*, 12 (1): 31-36. doi: https://doi.org/10.1016/S0956-7135(00)00016-5.
Ruusunen, M., Surakka, A. N. U., Korkeala, H. & LindstrÖM, M. (2012). Clostridium tyrobutyricum Strains Show Wide Variation in Growth at Different NaCl, pH, and Temperature Conditions. *Journal of Food Protection*, 75 (10): 1791-1795. doi: 10.4315/0362-028X.JFP-12-109.
Schiraldi, C. & De Rosa, M. (2016). Mesophilic Organisms. In Drioli, E. & Giorno, L. (eds) *Encyclopedia of Membranes*, pp. 1-2. Berlin, Heidelberg: Springer Berlin Heidelberg.
Sedlar, K., Vasylkivska, M., Musilova, J., Branska, B., Provaznik, I. & Patakova, P. (2021). Phenotypic and genomic analysis of isopropanol and 1,3-propanediol producer Clostridium diolis DSM 15410. *Genomics*, 113 (1, Part 2): 1109-1119. doi: https://doi.org/10.1016/j.ygeno.2020.11.007.

Silvetti, T., Morandi, S. & Brasca, M. (2018). Growth factors affecting gas production and reduction potential of vegetative cell and spore inocula of dairy-related Clostridium species. *LWT*, 92: 32-39. doi: https://doi.org/10.1016/j.lwt.2018.02.014.

Stenfors Arnesen, L. P., Fagerlund, A. & Granum, P. E. (2008). From soil to gut: Bacillus cereus and its food poisoning toxins. *FEMS Microbiology Reviews*, 32 (4): 579-606. doi: 10.1111/j.1574-6976.2008.00112.x.

Storari, M., Kulli, S., Wüthrich, D., Bruggmann, R., Berthoud, H. & Arias-Roth, E. (2016). Genomic approach to studying nutritional requirements of Clostridium tyrobutyricum and other Clostridia causing late blowing defects. *Food Microbiology*, 59: 213-223. doi: https://doi.org/10.1016/j.fm.2016.05.013.

Taylor, R. H., Dunn, M. L., Ogden, L. V., Jefferies, L. K., Eggett, D. L. & Steele, F. M. (2013). Conditions associated with Clostridium sporogenes growth as a surrogate for Clostridium botulinum in nonthermally processed canned butter. *Journal of Dairy Science*, 96 (5): 2754-2764. doi: https://doi.org/10.3168/jds.2012-6209.

Thorberg, B. M., Danielsson-Tham, M. L., Emanuelson, U. & Persson Waller, K. (2009). Bovine subclinical mastitis caused by different types of coagulase-negative staphylococci. *J Dairy Sci*, 92 (10): 4962-70. doi: 10.3168/jds.2009-2184.

Tirloni, E., Bernardi, C., Ghelardi, E., Celandroni, F., Andrighetto, C., Rota, N. & Stella, S. (2020a). Biopreservation as a potential hurdle for Bacillus cereus growth in fresh cheese. *Journal of Dairy Science*, 103 (1): 150-160. doi: https://doi.org/10.3168/jds.2019-16739.

Tirloni, E., Stella, S., Bernardi, C., Mazzantini, D., Celandroni, F. & Ghelardi, E. (2020b). Identification and Pathogenic Potential of Bacillus cereus Strains Isolated from a Dairy Processing Plant Producing PDO Taleggio Cheese. *Microorganisms*, 8 (6): 949. doi: 10.3390/microorganisms8060949.

Valero, A., Olague, E., Medina-Pradas, E., Garrido-Fernández, A., Romero-Gil, V., Cantalejo, M. J., García-Gimeno, R. M., Pérez-Rodríguez, F., Posada-Izquierdo, G. D. & Arroyo-López, F. N. (2020). Influence of Acid Adaptation on the Probability of Germination of Clostridium sporogenes Spores Against pH, NaCl and Time. *Foods*, 9 (2). doi: 10.3390/foods9020127.

Walstra, P. & Jenness, R. (1984). *Dairy chemistry and physics*.

Walstra, P. W. J. T. M. G. T. J. (2014). Dairy science and technology.

Warda, A. K., Siezen, R. J., Boekhorst, J., Wells-Bennik, M. H. J., de Jong, A., Kuipers, O. P., Nierop Groot, M. N. & Abee, T. (2016). Linking Bacillus cereus Genotypes and Carbohydrate Utilization Capacity. *PloS one*, 11 (6): e0156796-e0156796. doi: 10.1371/journal.pone.0156796.

Yang, X., Wang, Z., Zhang, C., Wang, L., Pang, L., Zhang, D., Man, C. & Jiang, Y. (2021). Assessment of the production of Bacillus cereus protease and its effect on the quality of ultra-high temperature-sterilized whole milk. *Journal of Dairy Science*, 104 (6): 6577-6587. doi: https://doi.org/10.3168/jds.2020-19818.

Yeung, C. Y., Lee, H. C., Lin, S. P., Yang, Y. C., Huang, F. Y. & Chuang, C. K. (2006). Negative effect of heat sterilization on the free amino acid concentrations in infant formula. *European Journal of Clinical Nutrition*, 60 (1): 136-141. doi: 10.1038/sj.ejcn.1602279.

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway