Influence of the lactoperoxidase-system and N_2-flushing on raw milk microflora

Bachelor agro- and biotechnology
Specialization Foodmanagement
Thesis

Student: Quintyn Romanie
Academic year: 2014-2015
Lector: Ingrid De Man
Mentor: Patricia Munsch-Alatossava

University of Helsinki
Faculty of Agriculture and Forestry
Agnes Sjöberginkatu 2
FIN-00014 University of Helsinki
Finland
www.helsinki.fi/university/

KATHOLIEKE HOGESCHOOL VIVES
STUDIEGEBIED BIOTECHNIEK

WILGENSTRAAT 32
B-8800 ROESELARE
TEL. +32 51 23 23 30
FAX +32 51 22 82 58

BIO.ROESELARE@VIVES.BE
WWW.VIVES.BE

Foreword

During two months, I did research in the Microbiology lab of the dairy group at the University of Helsinki under supervision of post.doc. Patricia Munsch- Alatossava. Research was done on the influence of the lactoperoxidase-system and N_2-flushing on microflora present in raw milk, kept at 15-25°C. About this two months during traineeship, I wrote my thesis.

First of all, I want to thank post.doc. Patricia Munsch- Alatossava for the perfect accompaniment and support during this traineeship. My mentor helped me in many ways to complete a good thesis. Ms. Ingrid De Man organized this traineeship and corrected my thesis. Without the support and encouragement of my parents Didier Quintyn and Fabienne Martens, and my boyfriend Pieter Vlaeminck, it would have been impossible to complete these two months in Finland. They gave me the unique chance to take this challenge and to expand my experiences. It was an unforgettable experience and I am sure that this will help me to discover new interests in the food sector in the future.

Thanks a lot to my lector, mentor and family to complete this traineeship and thesis together with me.

Romanie Quintyn
June 2015

THESIS

Title

Research on the presence of different micro-organisms in raw milk treated by different antimicrobial systems at temperatures between 15°C and 25°C.

Abstract

Raw milk is a highly nutritious natural product with lots of positive characteristics. That's the reason why it's so sensitive for contamination of different species of microbiota. In developed countries, methods, milk equipment and storage-methods are developed. In this way refrigeration below 6°C after milking helps to hold the initial microflora low. In developing countries, there are not so many possibilities and transport happens by foot or by motorbike, for many hours at moderate temperatures. The refrigeration methods aren't developed at all and growth of the initial microflora is possible. The quality of the raw milk decreases before further heating treatments are possible in the factory. That's the reason why this research is done. Two methods are examined to help these countries. Research is done on the working and effectivity of the lactoperoxidase-system and N_2-flushing. The lactoperoxidase-system can be activated by adding two substrates thiocyanate and hydrogen peroxide. In this way an antimicrobial component is produced that has a bacteriostatic or bactericidal effect on different species. N_2-flushing happens in the headspace of the milk flasks and can also inhibit growth of the initial microflora. By examining these two methods, eight conditions are tested at 15°C or 25°C. There are four conditions without N_2-flushing and with the adding of one or both substrates thiocyanate or hydrogen peroxide, there is also a control-bottle without any substrate or N_2-flushing added. These are the four first conditions: control, raw milk+ thiocyanate+ hydrogen peroxide, raw milk+ thiocyanate, raw milk + hydrogen peroxide. The other four conditions are identically as described above but there is also N_2-flushing in these four conditions. On different counting points, samples are taken to follow the growth of the initial microflora's of the total aerobic colony count, *Lactobacillus* spp. and *Streptococcus* spp. and the effectiveness of both systems. The conditions with one or both substrates added AND N_2-flushing show lowest results in growth but the condition with both substrates thiocyanate AND hydrogen peroxide added AND N_2-flushing in the headspace is the best condition to hold the initial microflora's low at each counting point. So the combination of the two methods (adding thiocyanate and hydrogen perodixe + N_2-flushing) is the best. The condition with both substrates thiocyanate and hydrogen peroxide added is even one of the most effective conditions. There is growth inhibition of the initial microflora for 24 hours at 15°C, and for 11-12 hours at 25°C. Unless there is growth after a few hours, the condition with thiocyanate, hydrogen peroxide and N_2-flushing keeps the growth of the initial microflora lower than the other conditions. In this way, these two methods can be introduced in developing countries, where refrigeration isn't possible after milking.

Keywords

- Lactoperoxidase-system
- N_2-flushing
- Raw milk microflora

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Technical file internship

Name internship :	**University of Helsinki**

Adress :	**Agnes Sjöberginkatu 2**
	FIN-00014 University of Helsinki, Finland

Phone number:	**02341 58308**
GSM-number:	**040 9526429**
Fax:	**+358 9 191 58460**
E-mail:	**Patricia.munsch@helsinki.fi**
Head of service:	**Tapani Alatossava**
Stagementor:	**Patricia Munsch-Alatossava**
Sector:	**Faculty of Agriculture and Forestry**
Department:	**Faculty of Agriculture and Forestry**
Two relevant publications of the internship:	
Annex:	**/**

1 The university of Helsinki

The University of Helsinki is the largest University in Finland and is ranked as the 73th best of the world's 15000 universities. The Shangai-ranking examines every year different critical points to give the university a specific ranking on world scale. The ranking is based on the quantity and impact of scientific information published by the authors and the receiving of Nobel Prizes. It is also one of Europe's top multidisciplinary universities. The university's goal is to become one of the 50 best universities in the world. The University of Helsinki achieved its highest ranking in arts and humanities in 2014, with a 50th place (University of Helsinki, 2014), (Academic ranking of world universities, 2014).

1.1 Strategy

1.1.1 Mission of the University

'The University of Helsinki is the most comprehensive institution of research, edification and intellectual regeneration in Finland. It is a pioneer and a builder of the future.' (University of Helsinki, 2015)

1.1.2 Vision for 2020: Excellence for society

The University of Helsinki consolidates its position among the leading multidisciplinary research-intensive universities in the world. It actively promotes the wellbeing of humanity and a just society.

The vision is supplemented with long-term strategic objectives of key importance:

- The University of Helsinki ranks among the 50 leading universities in the world;
- The University of Helsinki is a responsible social force;
- The University of Helsinki is a thriving and inspiring community and;
- The University of Helsinki keeps its finances on a sustainable footing.

(University of Helsinki, 2015)

1.1.3 Strategic objectives for 2020 and key areas of development until 2016

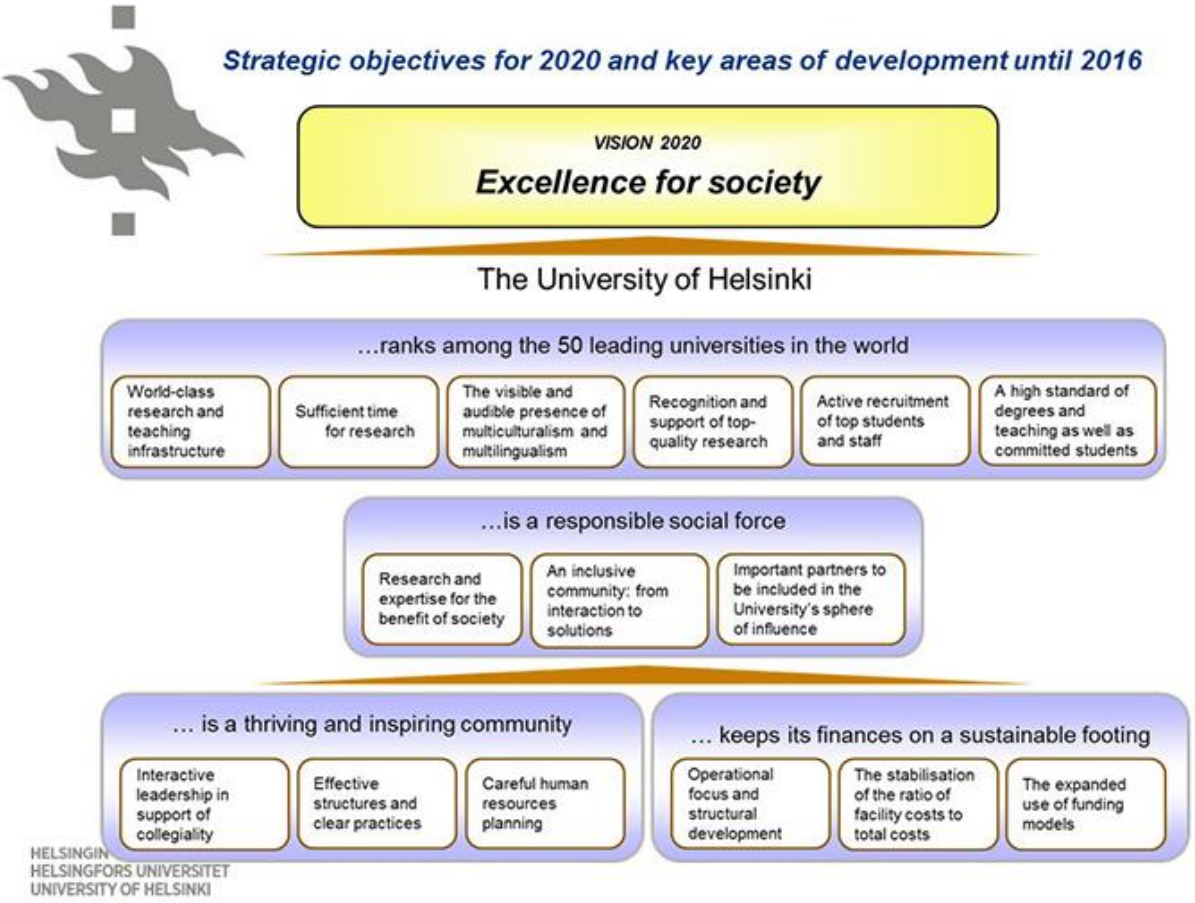


Figure 1: Strategic objectives for 2020 and key areas of development until 2016

(University of Helsinki, 2015)

1.2 Organisation

1.2.1 The Highest level

- University Collegium
  - 50 members
  - Decides on the number of Board of members
  - Determines the duration of the terms of office of the Board and its members
  - Approves the University's financial statements and the annual report
- The Board
  - Consists of 13 members
  - Highest decision-making body lead by the chair
- The rector
  - Directs the University's operations
- The chancellor
  - Promotes science and scholarship
  - Looks after the University's general interests

(University of Helsinki, 2015)

1.2.2 The second level

- The faculties
  - Led by the dean
  - Departments led by the head of department
- The independent institutes
  - 18 independent institutes
  - Focus on research, teaching or the provision of services
  - Led by a director and a board

(University of Helsinki, 2015)

1.2.3 The third level

- The faculty department and institutes

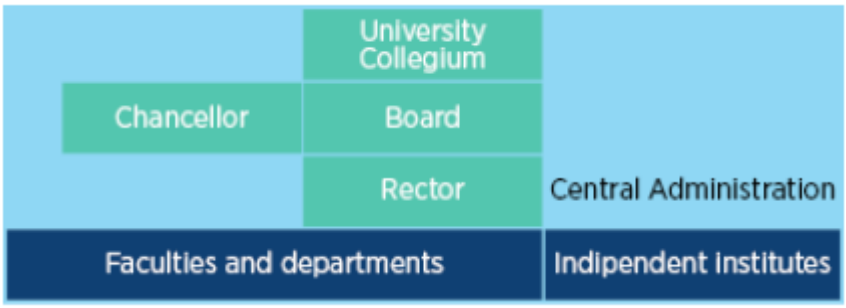


Figure 2: The faculty department and institutes

(University of Helsinki, 2015)

1.3 Faculties and departments

- Faculty of Arts;
- Faculty of Behavioural Sciences;
- Training Schools;
- Faculty of Law;
- Faculty of Social Sciences;
- Faculty of Theology;
- Swedish School of Social Science;
- Faculty of Science;
- Faculty of Medicine;
- Faculty of Biological and Environmental Sciences;
- Faculty of Agriculture and Forestry;
- Faculty of Veterinary Medicine and;
- Faculty of Pharmacy.

As listed above, the University of Helsinki contains 11 different faculties in four campuses in Helsinki and in 9 other localities in Finland. Furthermore, the University of Helsinki accommodates several independent research-oriented institutes, campus units and research networks. It also facilitates to duties of a national authority e.g. the Finnish Food Safety Authority (University of Helsinki, 2015).

1.4 The University's finances

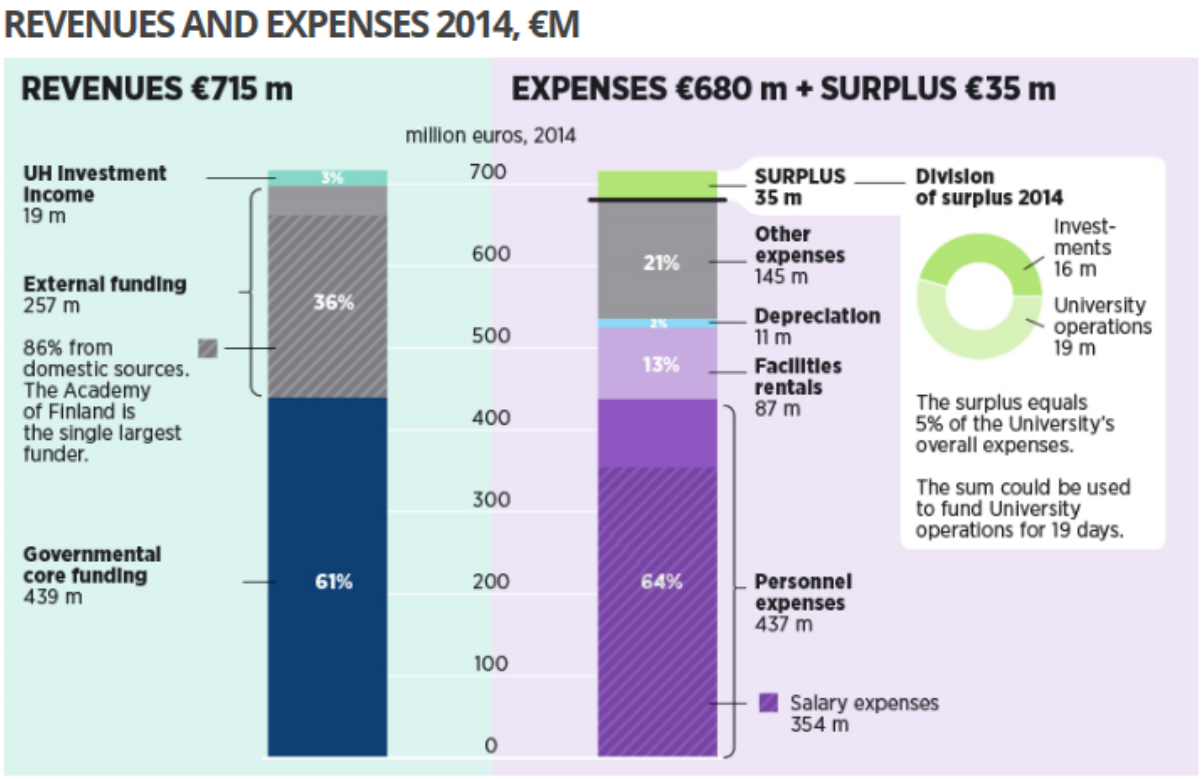


Figure 3: The University's finances

In 2014, the University of Helsinki reported expenses of €680 million and revenues of €715 million. This amounts to a surplus of €35 million. Most of the University's money, 61% comes from Governmental core funding, approximately €439 million. 36% of €257 million comes from external funding e.g. The Academy of Finland, which is the single largest funder. The other 3% or €19 million comes from investments profits (University of Helsinki, 2015).

The biggest cost item of the University of Helsinki are personnel expenses of operating expenses, which result in 64% of the expenses or €437 million. The other €326 million are divided into facilities rentals (€87 million), depreciations (€11 million) and other expenses (€145 million) (University of Helsinki, 2015).

1.5 Some facts about the University of Helsinki

- Founded in 1640;
- Budget: over 600 million euro;
- Over 30 international Master's Degree Programmes;
- The biggest university in Finland with over 35000 students, 2000 of whom are international;
- 11 faculties and 4 campuses within the Helsinki area;
- Total staff of 8590;
  - 4800 researchers, professors and lecturers, of whom 768 are foreign;
  - 3770 other staff, 113 foreign;
  - 59% women, 41% men.
- 5800 awarded degrees per year;
- Ranked in the top 100 in the world and top 25 in Europe and;
- A founding member of the League of European Research Universities (LERU).

(University of Helsinki,2015)

1.6 The Viikki Campus

Viikki Campus is a part of the University of Helsinki and it is an important concentration in the field of biosciences. The Viikki Campus is also called 'the green campus'.

The Campus is home to two independent research institutes of the University of Helsinki, the Viikki Science Library and four faculties:

- The Faculty of Agriculture and Forestry;
- The Faculty of Biological and Environmental Sciences;
- The Faculty of Pharmacy and
- The Faculty of Veterinary Medicine.

The Viikki Campus is one of the biggest bioscience centres in Europe with approximately 6500 students and 1800 staff members (University of Helsinki, 2015).

1.6.1 Location of the Viikki Campus

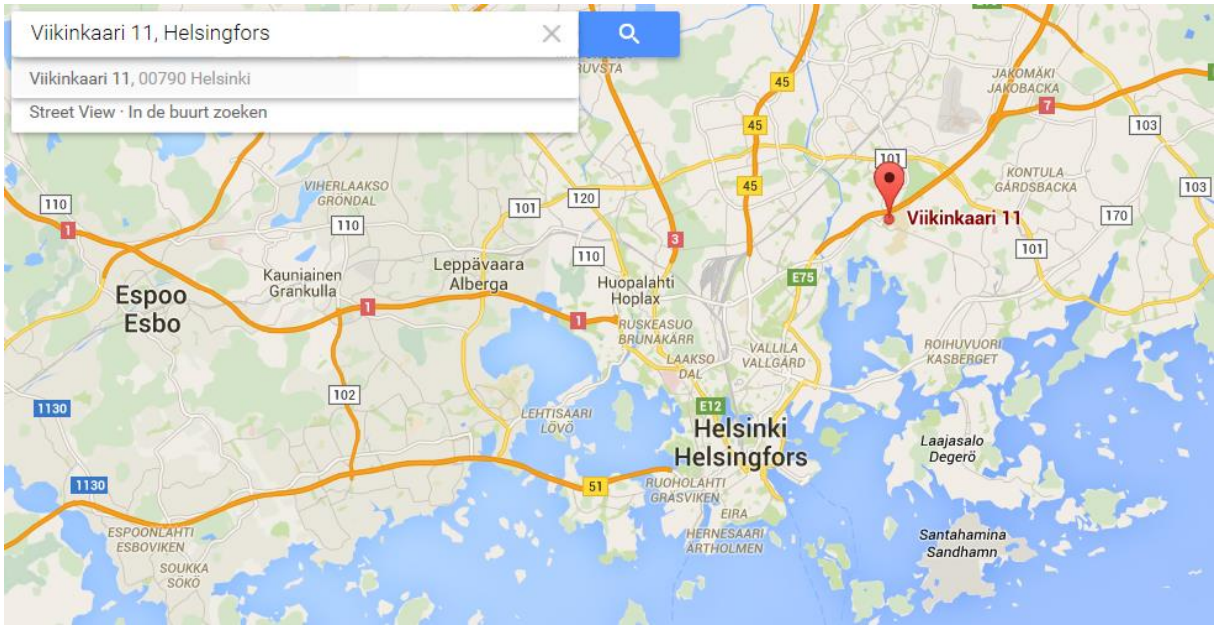


Figure 4: Location of the Viikki Campus

(Google Maps, 2015)

1.6.2 Faculty of Agriculture and Forestry

The Faculty of Agriculture and Forestry is the 5th largest Faculty in the University of Helsinki. It has approximately 2700 students, 480 post-graduate students, 500 staff members and 4 departments:

- The Department of Agricultural Sciences;
- The Department of Forest Sciences;
- The Department of Food and Environmental Sciences and
- The Department of Economics and Management.

(University of Helsinki, 2015)

Mission

' Faculty of Agriculture and Forestry promotes the responsible use of renewable natural resources through high-quality applied research, active participation in public debate and education in life sciences and business.' (University of Helsinki, 2015)

Vision

'The Faculty aims to be an internationally recognised research and teaching unit that enjoys a solid reputation for its scientific expertise and is regarded as an attractive cooperation partner.' (University of Helsinki, 2015)

Values of Faculty of Agriculture and Forestry

'We share the essential values of critical analysis, creativity and the pursuit of truth with the university. In addition, our values include respect for human well-being and the appreciation of nature and the environment.' (University of Helsinki, 2015)

Microbiology lab of the dairy group

The research for this thesis was done at the Microbiology lab of the dairy group under supervision of Professor Tapani Alatossava. At this lab, research is done on different groups of dairy: icecream, cheese, milk, etc.

There are four people working at this lab: one professor, one lector and two post-doctoral researchers. These four people are working in two teams. Professor Tapani Alatossava and post.doc. Patricia Munsch- Alatossava are working together. Lector Pekka Varmanen and post.doc. Kirsi Savijoki are working together at the lab.

Professor Tapani Alatossava is specialist in Lactic Acid Bacteria and bacteriophages. Post.doc. Patricia Munsch-Alatossava is specialist in the research on microflora present in raw and pasteurized milk. But she also does research on *Chlostridium tyrobutyricum* in cheese and antibiotic resistance. Pekka Varmanen is a lector and post.doc. Kirsi Savijoki is specialised in the mastitis bacteria and the Lactic Acid Bacteria.

At this lab, research is done on different dairy subjects, to make and write scientific publications or commissioned by the industry.

Intent

This thesis is written at the University of Helsinki in the Department of Forestry and Agriculture, in the Dairy Technology Group under supervision of Professor Tapani Alatossava. Research is done on the activation of the lactoperoxidase-system and N_2-flushing in raw milk, obtained after milking. Influences of these antimicrobial systems will be examined on the initial microflora of the total aerobic colony count, *Lactobacillus* spp. and *Streptococcus* spp.

The intention of this thesis is formed by the needs of good and effective antimicrobial systems to control the initial microbial counts of raw milk in developing countries. In developed countries, like the European countries, refrigeration directly after milking is normal and results in lower microbiological counts, by controlling optimal storage and transport conditions before further treatments in the factory. In these countries, milk production methods, equipment and milk-storage have improved over time. In developing countries, like the African countries, storage and transport methods aren´t developed and are sometimes very low. Because of that, refrigeration at 4°C after milking isn´t possible in many cases. In developing countries, transport of the raw milk happens in a lot of cases by foot or by moped, across bad roads. In that way, it takes more time to bring the raw milk to the factory at incorrect temperatures. The initial microflora increases a lot during transport and storage, before further treatments in the factory. As a result, the initial quality of the raw milk is gone and further treatments can´t optimize this.

This thesis has the intention to respond to following questions and problems:

- Doing research on the effectivity, activation and influence of the lactoperoxidase-system on the initial microflora present in raw milk by adding substrates thiocyanate or H_2O_2. Different experiments will be done at 15°C and 25°C, on different counting points, to examine the working of this first antimicrobial system.
- Doing research on the effectivity and influence on the initial microflora of N_2-flushing in the headspace of the storage of raw milk. Different experiments will be done at 15°C and 25°C, on different counting points, to examine the working of this second antimicrobial system.
- A comparison will be made between these two antimicrobial systems, to detect which system is most effective to use or to apply in developing countries, where refrigeration is not possible immediately after milking. Furthermore there will be checked if a combination of these two systems is possible or more effective than when they are used seperately.

2 Introduction/literature study

The subject of this literature study is the study of Raw Milk Microflora that live at temperatures between 15-25°C, by using different defense mechanisms such as lactoperoxidase and N_2-gas to preserve the raw milk. Research was done on the cause, the source, the detection and influences of storage and different treatments on these microorganisms in raw milk.

2.1 Milk

Definition: Milk is a highly nutritious food that can be obtained from a variety of animal sources such as cows, sheep, buffalo and goats. It is a complex biological fluid secreted in the mammary glands of mammals (Wareing *et al.,*2009). But also humans produce milk after pregnancy (Quigley *et al.,*2013).

Function: To meet the nutritional needs of neonates of the species from which the milk is derived (Leatherhead Food International*,*2009).

Average composition:

- Proteins (3.2-3.5%)
- Fats (3.7-3.9%)
- Carbohydrates (4.8-4.9%)
- Vitamins
- Minerals
- Essential amino acids
- Water (87%)

There is 0.7% ash (Leatherhead Food International*,*2009).

Raw milk has a neutral pH of about 6.5 and a high water activity (Quigley *et al.,*2013).

2.2 Cause of the presence of microorganisms in raw milk

Milk has a high nutritional content, which can support a rich microbiota. Some of the nutrients (written above) are directly available to all microorganisms. Others are provided following the metabolism of major components by specific populations to release components and metabolites that are used by others (Quigley *et al.,*2013).

But also the presence of antibiotic residues in milk can introduce the development of resistance of all types of micro-organisms. When pathogenic bacteria develop resistance, this causes problems in raw milk where the microbiological growth increases (Quigley *et al.,*2013).

2.3 Sources of microorganisms

There are three basic sources of microbial contamination of milk:

1. Micro-organisms present in raw milk from nature.
2. Micro-organisms from the exterior of the teats and udder.
3. Micro-organisms from the milk handling and storage equipment. These micro-organisms enter the milk from the environment.

2.3.1 The initial microflora of raw milk

Milk in healthy udder cells is thought to be sterile. The number and different types of microorganisms in milk immediately after production (= intitial microflora) directly reflect microbial contamination during production, collection and handling (Chambers, 2002).

When milk leaves the udder, there is an initial microflora present in the raw milk from nature. How this initial microflora develops, depends on the storage temperature and the elapsed time after collection. In normal circumstances, raw milk is stored at temperatures below 4°C. This temperature delays the bacterial multiplication for at least 24 hours. The microflora count at this moment, is the initial microflora (Chambers, 2002).

In developed countries, refrigeration immediately after milking is normal and results in lower microbiological counts. In these countries, milk production methods, equipment and milk-storage have improved over time. In developing countries, milk treatments and production methods are under developed. In these countries, there is not enough money to have the same facilities to produce in controlled circumstances. So refrigeration at 4°C isn't possible in many cases. In this way, the initial microflora increases a lot during storage and transport to the factory (Chambers, 2002). During this internship, different methods were examined to control the initial microbial count in countries where storage conditions aren't developed.

2.3.2 Contamination from the exterior of the teats and udder

Although milk produced from the mammary glands of healthy animals is initially sterile, microorganisms are able to enter the udder trough the teat duct opening. (Leatherhead Food International,2009) The bovine teat surface can also contain a large number of different microorganisms. Some of them can be identified such as *Solobacterium* and *Clavibacter.* Others are unidentified bacteria. Note that some of them are important to the flavour and colour development in cheese. Then again other bacteria have a rather negative influence on the milk quality. The composition of the microbiological community on the teat surface varies quantitatively and qualitatively from farm to farm. The bedding material, milking machines and the hygiene of the environment are different on each farm and cause differences in microbiological compound of the teat surface. When animals are fed indoors or outdoors, also plays a role in the microbial compound of the teat surface (Quigley *et al.,*2013).

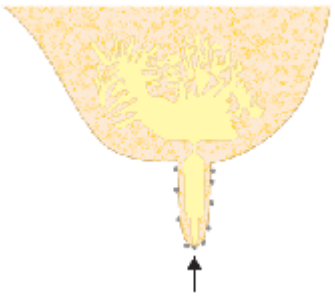


Figure 5: Contamination trough the duct opening

Gram-positive cocci, streptococci, lactic acid bacteria (LAB), *Pseudomonas* spp. and yeast are most frequently found in milk drawn aseptically from the udder (Leatherhead Food International,2009).

When the mammary is infected with mastitis, the mammary tissue is inflamed. In this case, large numbers of microorganisms and somatic cells are usually shed into the milk. The

organisms involved in mastitis are usually not able to grow in refrigerated milk, but they are able to survive and their presence may be a cause of concern for health (Leatherhead Food International,2009).

When mastitis is present in a clinical form, macroscopic changes to the milk and udder are readily detectable at the time of milking. There is also subclinical mastitis, which is more common. When subclinical mastitis is present, milk and udder appear normal. Subclinical mastitis can only be diagnosed by testing milk samples. This results in the presence of pathogenic bacteria and increased somatic cell count (Chambers, 2002).

It is notably *Staphylococcus aureus* and *Streptococcus agalactiae* that colonize the teat duct, particularly in the region of the teat orifice. But also *Streptococcus uberis, Streptococcus dysgalactiae, Escherichia coli, Mycoplasma, Corynebacterium pyogenes, Iydroteae iritans* and *Corynebacterium bovis* can cause mastitis. The colonization of the teat orifice can persist for many weeks without the penetration of the bacteria to the teat sinus. Once penetration has occurred, the adaptation to the new environment takes place followed by growth and the release of toxins. This results in the condition known as mastitis (Chambers, 2002).

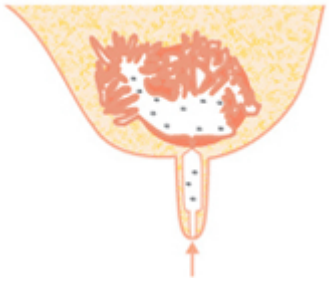


Figure 6: Contamination by mastitis

Consequently, a good animal husbandry and effective cleaning and disinfection of udders prior to milking are important in minimising contamination (Leatherhead Food International,2009).

2.3.3 Contamination from the milk handling and equipment

The milking equipment and bulk storage tanks make a contribution to the psychrotrophic microflora of raw milk when they are not good sanitised. The equipment and contaminated air are also sources of contamination when they are not good controlled and observed. When milk residues stay on surfaces, in joints and rubber seals, this can support the growth of psychotrophic Gram-negative organisms such as *Pseudomonas.* But also the growth of the spore-forming *Bacillus* and *Clostridium* is possible (Leatherhead Food International,2009).

All these organisms can be removed by effective and good cleaning and disinfection on regular times. But this has to be controlled very well because when they are forgotten, organisms can build up as biofilms in poorly cleaned equipment. And in this case, they are protected from cleaning and disinfection (Leatherhead Food International,2009).

But also the farm water, the farm workers and airborne microorganisms can be a source of contamination (Leatherhead Food International,2009).

Water used in the milk production process should have a good quality. The water must be from an approved source, free of pathogens and fecal contamination.

Fecal contamination is typically associated with the gut microorganisms. *Bacteroides* and *Prevotella,* but also coliforms, fecal streptococci and clostridia are examples of fecal contamination (Quigley *et al.,*2013).

Next to that, some microorganisms are present in milk, but not detected in the farm environment such as *Lactococcus* and *Lactobacillus.* There are also microorganisms detected in the farm environment but not in de milk (Quigley *et al.,*2013).

The trends are affected by temperature and the length of storage. These factors play a role in the numbers of microorganisms presented in raw milk (Quigley *et al.,*2013).

2.4 Roles of microorganisms

Microorganisms in milk can play many different roles, they can even be good or bad (Quigley *et al.,*2013).

1. Facilitating dairy fermentations (for example *Lactococcus, Lactobacillus)*
2. Causing spoilage (for example *Pseudomonas, Bacillus* or other spore-forming or thermoduric microorganisms)
3. Promoting health (for example lactobacilli and bifidobacteria)
4. Causing disease (for example *Listeria* and *Salmonella)*

2.4.1 Microorganisms facilitating dairy fermentations

Some bacteria can contribute subsequently to natural fermentations. These microorganisms are so successful that they are isolated from milk and function as starters in products to confer desirable traits on fermented products. In this case microorganisms are re-introduced in pasteurized milk (Quigley *et al.,*2013).

2.4.2 Microorganisms causing spoilage

Certain microorganisms such as *Pseudomonas* and *Bacillus* can cause spoilage of the milk by causing abnormal and bad taste, off-flavours, rancidity, ropiness, coagulation and colour changes of the milk. When spoilage dominates the milk, the milk can't be consumed anymore because of the bad quality. Spoilage may be the result from either the growth of psychrotrophic thermoduric organisms that survive pasteurisation or post-pasteurisation contamination by psychrotrophs (Leatherhead Food International*,*2009).

2.4.3 Health-promoting microorganisms

Some bacteria present in raw milk have a health-promoting influence. These bacteria are sometimes isolated from milk, to be added to other products. Isolates are able to survive bile juice, to tolerate gastric acid conditions and to adhere to intestinal cells in the human body. The health-promoting bacteria isolated from milk, are commonly referred as 'probiotics'. Probiotics are bacteria, which administered in adequate amounts, confer a health benefit on the host. Examples of probiotic bacteria are *Lactococcus lactis*, *Leuconostoc, Enterococcus* and *Streptococcus* isolates (Quigley *et al.,*2013).

2.4.4 Pathogenic bacteria causing disease

Certain bacteria can cause illnesses of varying severity. Normally, pathogenic microorganisms are removed by pasteurisation. When they are not removed, in case of raw milk or consuming raw milk cheeses, they can cause a serious health risk. Examples here are *Listeria* spp., *Salmonella* spp and *Campylobacter* spp. These are not able to survive the pasteurisation but they are present in raw milk and raw milk cheeses. Typical symptoms of

illness are fever, vomiting, diarrhea, nausea and in extreme cases death (Quigley *et al.,*2013).

2.5 Possible microorganisms

2.5.1 Psychrotrophic microorganisms

Psychrothropic microorganisms live at temperatures between -5°C and 35°C, but the optimum temperature for growth is about 25°C. Some microorganisms of these category have a good influence on milk and the subsequent products, others have only negative characteristics (Quigley *et al.,*2013).

These microorganisms have become more important for the shelf life of heat-treated dairy products because of the development of these bacteria during prolonged refrigerated storage of raw milk on the farm and the dairy plant (De Jonghe *et al.*,2010).

From values of 10^6-10^7/ml of psychotrophic germs, there are enough enzymes produced to detect deviations in the raw milk quality (De Jonghe *et al.*,2010).

Enterococcus

Enterococci occupy a wide range of ecological niches that include the gastrointestinal tracts of humans and animals. Depending on the class of Enterococci, they have a different function. Enterococci can be starter cultures (for example *Enterococcus faecalis)*, probiotics (for example *Enterococcus faecium)*, pathogenic organisms (for example *Enterococcus faecalis*= sometimes pathogenic*)* but they can also cause spoilage (Quigley *et al.,*2013).

Characteristics:

- They can survive adverse conditions;
- Can survive high-temperature;
- Can survive high-salinity environments;
- Can survive refrigeration;
- They can grow on different substrates and in many conditions.

Enterococci can survive pasteurisation, so they can be present both in raw milk and pasteurised milk, but also in the subsequent products. Some enterococcal species are often an important component of the natural cultures involved in fermentation. They contribute to ripening, flavour and taste, due to their proteolytic activity and ability to hydrolyse milk fat (Quigley *et al.,*2013).

Enterococcus faecalis for example has a negative influence on humans' health. This species contributes to human infections (Quigley *et al.,*2013).

Listeria monocytogenes

Listeria monocytogenes is a psychrotrophic microorganism that lives at refrigeration temperatures, but it can grow until a temperature of 45°C. The optimum temperature of this pathogen is between 30 and 37°C, but it still grows at temperatures between 15 and 25°C, which is important. The growth will be more slowly at temperatures lower than 30°C, but *Listeria monocytogenes* stays present in raw milk (Leatherhead Food International*,*2009).

Listeria monocytogenes is a facultative anaerobe. This bacteria can survive (once present in the milk, the initial quantity stays, there is no growth) or grow (the initial quantity of this microorganism increases which causes problems in quality, spoilage, etc.) in a lot of

circumstances such as salty environments, in sour and dry foods. Anyway, this pathogen isn't able to survive the pasteurization, so it can be found in raw milk and raw milk cheeses. When it's detected in pasteurized milk, cross-contamination after pasteurization can happen (Bekaert,2013).

Characteristics:

- In ideal conditions, growth is possible at pH values below pH 5;
- At refrigeration temperatures, the lowest limit for growth is pH= 5.2;
- Tolerant for high NaCl concentrations;
- It grows best at an aw-value ≥0.97;
- The target value is absence in 25 grams.

(Leatherhead Food International,2009)

Bacillus cereus

Bacillus cereus is an aerobic or facultative aerobic, Gram-positive spore-forming bacteria that lives under psychrotrophic conditions. This microorganism grows at temperatures within the range of 4-37°C, so this bacteria can proliferate perfect at refrigeration temperatures but also at room temperatures. The presence in raw milk is possible (Bekaert, 2013).

This pathogen is a food intoxicant that is able to produce enterotoxins. But only at high numbers, there is danger for intoxication. The advantage here is that vegetative cells of *Bacillus cereus* are destroyed by pasteurization. But the negative thing is that spores of this pathogen are awakened by the pasteurization process and they survive. The spores become activated and produce emetic toxins. Therefore the germinated spores can still cause intoxication in pasteurized milk and this is the dangerous part of *Bacillus cereus* in raw milk. This toxin is stable in the pH-range 2-11 and resistant to heating till 126°C for 90 minutes. So destroying the vegetative cells is useless, because of the production of the intoxicating enterotoxins (Leatherhead Food International,2009).

There are different enterotoxins that can be formed by active growth or the growth of spores. One of them is the diarrheal enterotoxin. This is a protein produced during active growth and is unstable at pH values <4.0 or >11.0. This enterotoxin however is heat-sensitive and is already destroyed at 56°C for 5 minutes (Leatherhead Food International,2009).

Characteristics:

- Capability to grow at pH values between 4.3 and 9.3;
- The optimum water activity to grow is 0.95, but the pathogen can still grow at water activity of 0.91.

(Leatherhead Food International,2009)

Detection of *Bacillus cereus*:

The colony-forming units of the species *Bacillus cereus*, are the units that develop for each gram or ml after 18-30 hours incubation at 30°C on a specific prepared medium. The medium (MYP agar) consists of three ingredients: a basic medium (=Cereus selective agar base,Merck), a polymyxine-solution and an egg yolk-emulsion (Mossel,1990).

Each part of the medium has a certain function:

- Inhibitor:
  The polymixine-solution is an antibiotic used to reach a certain selectivity. Polymixine inhibits the growth of competitive bacteria, notably Gram-negatives and cocci.;
- Indicator:

The lecithine present in the egg yolk ensures that *Bacillus cereus* forms wide, white halos, which makes them identifiable. *Bacillus cereus* is lecithine positive. This species produces phospholipase and it's the phospholipase that has an influence on the egg yolk and causes a white halo. All other *Bacillus* species are lecithine negative and don't form white halos.;

- Differential indicator:
  The mannitol present in the basic medium ensures the difference between *Bacillus cereus* and other *Bacillus* species, because *Bacillus cereus* doesn't corrode the mannitol. *Bacillus cereus* is mannitol negative and causes pink colonies because this species doesn't ferment the mannitol.

(Mossel,1990)

After incubation, the colonies have to be counted. The typical *Bacillus cereus* colonies don't color the agar yellow and have a white circle around them. The colonies of *Bacillus cereus* are pink. Other *Bacillus* species that are mannitol positive, cause yellow colonies because of the fermentation of the mannitol (Mossel,1990).

***Pseudomonas* spp.**

Pseudomonas spp. are commonly found in raw milk and are the most common cause of milk spoilage. The most commonly detected species in milk are *Pseudomonas fluorescens, Pseudomonas gessardii, Pseudomonas fragi* and *Pseudomonas lundensis* (Quigley *et al.,*2013). These microorganisms can still grow at low temperatures of 4°C, so they proliferate during the storage of raw milk.

The bacteria belonging to the genus of *Pseudomonas* are capable of producing thermoresistant extracellular proteases and lipases, which can cause spoilage and structural defects in pasteurized and UHT milk. The bacteria may be destroyed by pasteurization but the enzymes are resistant to heat treatments (De Jonghe *et al.*,2010).

Pseudomonas enter the raw milk via biofilms in the milk tanks, contaminated water and also via soil (De Jonghe *et al.*,2010).

Detection total *Pseudomonas* plate count:

The total *Pseudomonas* plate count is determined by plating on CFC agar (= Pseudomonas selective agar+ CFC Supplement). The plates are incubated during 48 hours at 25°C (NIZO,2010).

The medium used to determine the quantity of *Pseudomonas* in milk, consists of a basic medium and a CFC-selective supplement. Each part has a certain function:

- Inhibitor:
  The CFC-supplement contains centrimide, cephaloridine and fucidine and delivers a selective medium where only *Pseudomonas* species can grow.;
- Indicator:
  The basic medium consists of *Pseudomonas* agar base. To promote the pigment production, magnesiumchlorid and potassium sulphate are added to the basic medium. Colonies are typical blue-green or brown. Fluorescence also appears for typical *Pseudomonas* species.

(Mossel,1990)

Different dilutions are made to ensure that surely one of the dilutions delivers colonies between the 15 and 150. From each dilution, you have to pipet 0.1 ml in the middle of the plate.This quantity has to be spread on the plate by a sterile Drigalski-spatula. For each examined dilution, there have to be made 2 CFC-agar plates (Mossel,1990).

After incubating, the colony forming units have to be counted on the plates (Mossel,1990).

2.5.2 Mesophilic microorganisms

Mesophilic microorganisms live at temperatures between 10°C and 45°C, but the optimum temperature for the growth of these microorganisms is 35°C.

Escherichia coli O157

E.Coli O157 isn't normally present in raw milk. The presence of this microorganism indicates faecal contamination. This bacteria is a microorganism that lives in the intestines of humans and animals. So when there is contamination of milk with *E.Coli O157,* there has been contact between the raw milk and faecals of the cows. In this way, *E.Coli O157* is an indicator of the hygiene (Leatherhead Food International*,*2009).

Escherichia coli 0157 is a facultative anaerobe but can survive under aerobic and anaerobic conditions. Besides this, the pathogen grows at temperatures between 7°C and 45°C, with an optimum temperature of 37°C. So the possibility to grow between a temperature of 15-25°C is present. The positive thing here is that the bacteria isn't heat-resistant. *E.coli* can only be found in raw milk (Leatherhead Food International*,*2009).

Characteristics:

- The minimum pH for growth under optimal conditions is 4.0-4.4;
- The microorganism is acid-tolerant;
- It can tolerate certain drying processes;
- *E.Coli* grows well at NaCl concentrations up to 2.5%.

(Leatherhead Food International*,*2009)

Lactic Acid Bacteria (LAB)

Lactic acid bacteria are a group of Gram positive bacteria and are non-respiring and non-spore forming. The bacteria are able to convert natural sugars (carbohydrates) into lactic acid by fermentation. When all sorts of milk are left to stand, acidity increases due to the presence of lactic acid bacteria. In this case, when you store raw milk at a temperature between 15 and 25°C, the milk will be sour caused by the population of growing LAB's (Sharma *et al.,*2013).

Lactic acid bacteria can be found in habitats with a rich nutrition supply such as plant material, dairy products, beverages and juices, where the presence of these bacteria is associated with poor hygiene, inconsistent quality presentation and short shelf life due to acidity development (Sharma *et al.,*2013).

But these microorganisms are also used as starter cultures in the food production, where they are responsible for the fermentation of different food products (Sharma *et al.,*2013).

***Lactobacillus* spp.**

Lactobacillus spp. are member of the Lactic Acid Bacteria-family. They are facultative anaerobe and can be found in rich carbohydrate-containing niches including those associated with plants, animals, silage and raw milk. They have proteolytic activity and ability to produce aroma compounds and exopolysaccharides that contribute to the quality and nutritional value of dairy products. So these bacteria play an important role in the characteristics, quality, flavor and taste of dairy products (Quigley *et al.,*2013).

These microorganisms are present in the initial microbiota of raw milk. *Lactobacillus bulgaricus* and *Lactobacillus thermophilus* are the most important species in this family (Quigley *et al.,*2013).

But there are also other lactobacilli in raw milk which increase in number during the manufacturing of dairy products. These lactobacilli can become dominant during the ripening of cheese (Quigley *et al.,*2013).

Detection of *Lactobacillus* spp.

The detection of *Lactobacillus* spp. is possible on MRS-agar. This medium is based on the formulations of deMan, Rogosa and Sharpe (MRS). Incubation should happen in an anaerobic jar during 3 days at 30°C or 2 days at 32°C.

Each part of the medium has a certain function:

- Carbon source: enzymatic digest of animal tissue;
- Nitrogen source: beef extract;
- Vitamin source: yeast extract;
- Inhibitor: sodium acetate;
- Fermentable carbohydrate: dextrose;
- Buffering agent: potassium phosphate.
  (Acumedia, 2011)

***Streptococcus* spp.**

Streptococcus spp. are microorganisms, part of the family of the Lactic Acid Bacteria (LAB). They are Gram-positive bacteria and are oxidase-negative and catalase-negative. These species are facultative anaerobe so they have to be incubated in an anaerobic jar. Many genera of streptococci are pathogenic, although *Streptococcus thermophilus* is an important starter culture in dairy products such as cheeses. This species has a GRAS-status. Despite 80% of the genome sequencing of *Streptococcus thermophilus* being similar with the pathogenic relatives, this species plays an important role in the texture, rheological properties and flavors of fermented milk products, particularly yoghurt (Quigley *et al.,*2013).

Besides a few species of the streptococci that play a role in raw milk and raw milk products, there are a lot of pathogenic streptococci. Many streptococci are associated with mastitis infection. *Streptococcus uberis, Streptococcus agalactiae* and *Streptococcus dysgalactiae* are pathogens that cause bovine mastitis worldwide (Quigley *et al.,*2013). That's also written above in the part about mastitis.

Streptococcus bovis is an opportunistic human pathogen, which is often associated with infections in immunocompromised patients or patients with cancer (Quigley *et al.,*2013).

Streptococci can also be find in fermented foods (Quigley *et al.,*2013).

Detection of *Streptococcus* spp.

M17 agar-medium is used for the isolation and the enumerating of lactic streptococci. Lactic streptococci are acid-producing bacteria so they need complex growth media for optimum growth. In a culture medium without an adequate buffering system, the pH decreases and affects growth. M16 agar-medium lacks of a good buffering system, whereby the M17-agar

medium is created. This medium consists of disodium-β-glycerophosphate as buffering component (BD,2015).

Each part of the medium has a certain function:

- Buffering component and inhibitor:
  Disodium-β-glycerophosphate buffers the medium when acid is produced from fermentation of lactose, but this component also suppresses the growth of *Lactobacillus bulgaricus* and selectively isolates *Streptococcus thermofilus* (BD, 2015).

2.6 Impact of storage and different treatments on the microbiology of raw milk

2.6.1 Cold storage

Milk is stored at refrigeration temperatures of <7°C in each part of the collecting and further production. On the farm and on the processing site, the milk is transferred and stored in bulk storage tanks or silos, prior to processing. This has consequences for a lot of properties of the milk. One of them is the decrease of the microorganisms in the milk (Quigley *et al.,*2013).

The growth of most bacteria is restrained (=lower growth rate), with the exception of psychrotolerant microorganisms that proliferate under these conditions and cause milk spoilage. The milk spoilage is caused by the production of extracellular enzymes. Lipases and proteases are the most important. Lipases degrade milk fat causing rancidity. Proteases degrade casein producing a grey color and bitter off-flavors (Quigley *et al.,*2013).

One of the psychrotolerant bacteria is *Pseudomonas* spp., which is the most common cause of milk spoilage. These bacteria are the most important bacteria in raw milk stored at refrigeration temperatures, and are the predominant one's that can contain 70-90% of the microbiological population (Quigley *et al.,*2013).

Another bacteria population that increases in cold storage is *Listeria monocytogenes* (Quigley *et al.,*2013).

Yeasts and moulds also have an influence on milk spoilage (Quigley *et al.,*2013).

The number of microorganisms in raw milk can increase during the transport as a result of contamination of inadequately cleaned tankers or from the growth of psychrotrophic organisms such as *Pseudomonas* spp. Important to mention is that the degree of growth depends on the initial microbial load, storage time and temperature (Leatherhead Food International*,*2009).

2.6.2 Thermisation

A treatment commonly used in the milk sector is thermisation. This is a mild heat treatment at 57-68°C during 15-20 seconds and then rapid cooling to <6°C . This reduces the psychrotrophic microorganisms and prolongs the storage life of the milk for several days. But thermisation doesn't eliminate all microorganisms. Some vegetative pathogens can survive, such as *L.monocytogenes* (Leatherhead Food International*,*2009).

2.6.3 Addition of carbon dioxide

When carbon dioxide is added to raw milk, three mechanisms happen and inhibit the growth of microorganisms:

- Displacement of oxygen;
- Lowering of the pH of the milk due to the dissolution of carbon dioxide and formation of carbonic acid;
- Direct effect on the metabolism of microorganisms by inhibiting the production of enzymes.

(Leatherhead Food International*,*2009)

When carbon dioxide is added, the system reduces proteolysis and lipolysis, which is positive. But it also modifies the sensory properties of the milk and as written above, it causes acidification of the milk, which is negative (Munsch-Alatossava *et al.,*2009).

2.6.4 Pasteurization

Pasteurization is a heat treatment of raw milk for a certain time, with the aim to reduce the microbial load of milk in order to limit the number of spoilage microorganisms (such as *Pseudomonas spp.*) and pathogens. Typical milk pasteurization is a 'High-Temperature Short-Time' (HTST) pasteurization, by heating till 72°C for 15 seconds (Quigley *et al.,*2013).

It reduces the psychrotrophic and mesophilic populations (Quigley *et al.,*2013).

But this process also reduces the good microorganisms that typically contribute to desirable sensory properties associated with raw milk cheeses. So starter cultures that give these desirable flavours and aromas, are added to the milk postpasteurization (Quigley *et al.,*2013).

Besides that, pasteurization can encourage the activation of spores, which may be dominant in milk. This type of microorganisms enter the milk chain trough soil, silage and bedding material and are resistant to pasteurization. They can survive and grow at refrigeration temperatures and can survive the pasteurization. Examples of these spore-forming microorganisms are *Clostridium sporogenes* and *Bacillus cereus.* They cause spoilage in pasteurized milk and the subsequent cheeses by causing off-flavors and curdling. Even more they also produce toxins that cause food-poisoning and are dangerous for the food safety (Quigley *et al.,*2013).

2.6.5 UHT (Ultra High Temperature) or sterilisation process

This heating treatment eliminates all vegetative cells in the milk and the majority of spores. Only very heat-resistant spores can survive the sterilisation process. An example here is *Bacillus sporothermodurans* which can survive the UHT process and grow in the final product. But this organism is not pathogenic and doesn't change the quality of the milk (Leatherhead Food International*,*2009).

This heating process achieves 'commercial sterility'. The milk has a long shelf life and doesn't need any more refrigeration. But on the other hand, it creates organoleptic changes in the milk such as browning and descent of the nutrient content (Leatherhead Food International*,*2009).

Sterilisation happens at a temperature of about 120°C during 30 minutes. UHT processed milk involves preserving milk by holding it at a temperature of 140-150°C for 1-2 seconds. After this heat treatment aseptic filling into sterile cartons or other containers is necessary (Leatherhead Food International*,*2009).

2.6.6 Natural antimicrobial factor: lactoperoxidase

On this system, research will be done at 15°C and 25°C.

Description lactoperoxidase

Lactoperoxidase is an enzyme naturally present in raw milk. Bovine milk contains about 30 mg/l of lactoperoxidase. This enzyme has a certain bacteriostatic working when two chemical substrates are added to the milk. These substrates are thiocyanate and hydrogen peroxide (H_2O_2). To activate the lactoperoxidase-system, certain concentrations of these substrates must be added to the milk. 10 ppm (parts per million) of thiocyanate must be added and 8.5 ppm (parts per million) of H_2O_2. When the milk is kept at different temperatures, the bacteriostatic effect of lactoperoxidase will have effect for more or less hours and will control spoilage of the raw milk (FAO,2005).

Working of the lactoperoxidase-system

The lactoperoxidase-system is activated when thiocyanate and hydrogen peroxide (H_2O_2) are added to the milk. These two components are naturally present in raw milk in different concentrations depending on the feed given and on the species/breed of the animal. The concentrations of these components are not high enough to preserve the milk for a long time. The natural preservation of the milk is just two hours. To preserve the raw milk for a longer time, thiocyanate and hydrogen peroxide must be added in registrated quantities (described above) (FAO,2005).

Lactoperoxidase has the ability to oxidise the thiocyanate ion (SCN^-) in the presence of H_2O_2. The resulting chemical compound has an antibacterial effect in fresh raw milk. On the one hand, the system has a bacteriostatic effect (inhibiting bacterial growth) on species of normal gut flora such as *streptococci* and *lactobacilli.* On the other hand, the system has an bactericidal effect (killing bacteria) against gram-negative bacteria such as *Escherichia coli* and *Pseudomonas* (FAO,2005).

The reaction of the system occurs in the following way:

$SCN^- + H_2O_2$	---------->	$OSCN^- + H_2O$
(thiocyanate ion)+(hydrogen peroxide)	***lactoperoxidase***	(antibacterial compound)

The ‘Guidelines for the preservation of raw milk by use of the lactoperoxidase system’ was added in de Codex guidelines in 1991. In this Codex all the guidelines can be found to put up a good lactoperoxidase system at a specific temperature and in specific conditions. Following guidelines are important to set up a good lactoperoxidase-system (FAO,2005):

- The lactoperoxidase-system is a system to preserve the raw milk until further processing and pasteurization. Good hygienic practices and heat treatments of the milk are still necessary to hold on the quality of the milk. So the LP-system only has a preserving function of the raw milk during collection and transport (FAO,2005).
- This method can be used to preserve milk from different species. The effectiveness of this system depends on the initial microbial count and the species of the micro-organisms present in the raw milk. But also the temperature during the treatment plays an important role (FAO,2005).

Here the inhibitory effect of the system can be found at different temperatures to the extension of milk keeping quality (FAO,2005):

Temperature (°C)	Time (hours)
31-35	4-7
30	7-8
25	11-12
20	16-17
15	24-26
4	5-6 days

Table 1: Inhibitory effect of lactoperoxidase at different temperatures

Depending on the temperature, the preserving effect of the lactoperoxidase-system will be effective for more or less hours. At ambient temperatures around 30°C, which is normal in developing regions such as Africa and Asia, spoilage will be under control for 4-8 hours, which means that, farmers will have the time to transport the milk to the processing site (FAO,2005).

Important to mention here is that psychrotrophic bacteria such as *Pseudomonas* spp. and *Listeria monocytogenes* grow at low refrigeration temperatures. So when the milk is stored at <4°C, growth of psychrotrophic is still possible. In this case, the activation of the LP-system can delay the growth of these kind of microorganisms and the milk spoilage for several days (FAO,2005).

Reason of the system

Milk is a high nutritious food, full of important components for the human body, which support a healthy body. Otherwise, milk is also an income for a lot of farmers, who work each day to deliver the best milk to the processing sites (FAO,2005).

In this way, spoilage of the raw milk need to be controlled and minimalized to keep the quality and nutritious value of this natural product. A lot of treatments are possible to control spoilage and the bacterial count such as refrigeration, heat treatment, bactofugation, microfiltration and high-pressure treatments. These methods need expensive equipment, for which farmers and producers need to invest lots of money. In developing countries, where a lot of little farmers deliver the milk, investments in expensive equipment isn't possible (FAO,2005).

In developed countries, refrigeration of the milk after milking at <4°C is needed to control the initial microflora present in raw milk. In this way, the bacterial count in the raw milk can be controlled until processing and pasteurization. Bulk storage and transport happens at low temperatures, which is critical for a qualitive product at the start of processing. The cooled transport ensures that the raw milk arrives in a short time period at the processing plant.
In developing countries, production methods are not developed and still need a lot of progression to deliver good and qualitive raw milk to the processing sites. Refrigeration isn't possible in a lot of countries (for example the African countries) and transport doesn't happen in typical milk transporters. Transport of the milk happens by foot or by mopped, whereby the time between milking and processing or pasteurization is too long to deliver a safe product and to control the initial microflora. The temperature of <4°C isn't possible at all. In these countries, the bacterial count will be already too high at the start of the processing and therefor will lead to problems in the integral processing. Pasteurization is not enough to pull down the initial microflora (FAO,2005).

Therefore, the lactoperoxidase-system is developed and research is done on this system, which has an antibacterial working and can control the initial microflora in countries where refrigeration and cooled transport isn't easy or even possible (FAO,2005).

Effectiveness of the lactoperoxidase-system against different microorganisms

The lactoperoxidase-system has been used against a lot of different microorganisms such as HIV-1 virus, yeasts, moulds, protozoa, mycoplasma and a lot of other bacteria. This includes also the antibacterial effect against non-pathogenic starter cultures, spoilage bacteria and pathogenic bacteria that cause gastrointestinal infections in humans. These types of bacteria need to be controlled by this system in raw milk (FAO,2005).

Important to mention is that the system is more or less effective for different types of microorganisms. For some bacteria the system is bacteriostatic, for other types of bacteria the system has a bactericidal effect. The sensitivity of the bacteria for the lactoperoxidase-system depends on the different cell wall structures and inhibitory compounds generated by the organisms. There are for example many species that metabolically produce H_2O_2, which is accumulated in the growth medium. This H_2O_2 can activate the LP-system and lead to self-inhibition of the bacterial growth (FAO,2005).

A few species on which the system has a bacteriostatic of bactericidal effect (FAO,2005):

Bacteriostatic effect	**Bactericidal effect**
Streptococci	*Escherichia coli, including E.Coli O157*
Lactobacilli	*Salmonella* spp.
	Staphylococcus aureus

Table 2: The working of lactoperoxidase on different bacteria

Safety for humans and animals

The lactoperoxidase-system is a naturally protective system in the biology of animals. In that way it differs from other preservation systems such as pasteurization, UHT, etc. which are not natural. It functions as a protective antimicrobial mechanism in mucosal tissue, including the oral cavity and lung. In this way, the lactoperoxidase-system doesn't introduce substances into milk that are not normal human metabolites. This system is non-toxic when used according to the Guidelines of the Codex Alimentarius Commission (FAO,2005).

For the lactating animals it is also not toxic when the treatment is carried out only after the milk has been drawn from the teat (FAO,2005).

2.6.7 Potential of nitrogen gas (N_2) to extend the shelf life of raw milk

When raw milk is transported from the farm to the dairy plant, a cold chain below 6 °C, keeps the raw milk above the freezing point. The cold chain, together with a short transportation time and short storage times helps to control bacterial growth and to protect raw milk from the harmful effects of undesirable microbes. Even though the cold chain helps to control bacterial growth, psychrotrophic bacteria have the possibility to grow at these temperatures and they can produce heat-stable proteases and lipases. These enzymes have bad influences on the milk quality (Munsch-Alatossava *et al.,*2009).

As a result of the growth of psychrotrophic bacteria and their possibility to produce proteases and lipases, different additional control systems are developed in the food industry to improve the quality and safety of raw milk. CO_2-addition as described above in Addition of carbon dioxide and N_2-addition (nitrogen), or a combination of both, are modified atmospheres, which involve the replacement of air. Addition of CO_2 or N_2 shows an extension of the shelf life of raw milk. A disadvantage of CO_2-addition is that it modifies the sensory properties of the milk and adicification of the milk (Munsch-Alatossava *et al.,*2009).

When just N_2 is added, this inert gas overcomes the disadvantages of CO_2 use. N_2 not only prolongs the shelf life of raw milk, there is also growth inhibition of aerobes, psychrotrophs, enterobacteria, protease- and lipase-producers and *Listeria* spp. (Munsch-Alatossava *et al.,*2013).

The level of inhibition of the bacteria written above, depends on the storage temperature of the raw milk and on the N_2 flow rates.

During milk transportation, raw milk bulk tanks are open systems that allow gas exchange between the headspace of the tank and the external atmosphere. To use N_2 for extension of the shelf life of raw milk, an open system is created where N_2 is flushed trough the headspace of the milk-containing vessel (Munsch-Alatossava *et al.,*2013).

This system of N_2-flushing is still developing and will be examined during the thesis. Research will be done at the lab in samples of raw milk, coming from lorry tanks that deliver raw milk to Helsinki dairy Ltd (recently created at Viikki Campus).

To understand the working of this system, more information is noted in material and methods in 4.1.4. and 4.2.4. Experiments will be done on raw milk with N_2-flushing at 15°C and 25°C during this thesis. In the part results (5) and discussion and conclusion (6) more information about these experiments is noted.

3 Material and methods

3.1 Material

3.1.1 Lactoperoxidase-test at 15°C

- Fresh raw milk (4 x 75 ml)
- 4 sterilized bottles used to keep the raw milk during the test
- Sterile filters (Millex 0.22 µm Filter Unit)
- Magnetic rods
- Distilled water
- Saline water (NaCl 0.85%)
- Thiocyanate
- H_2O_2
- Pipets
- Rack to hold the sterile tubes
- Sterile test tubes
- Aceton
- Warm water bath at 15°C
- Cooling unit with ethanol
- Multi-place magnetic stirrer

- MRS (deMan-Rogosa and Sharpe)-growth medium
- M17- growth medium
- PCA (Plate Count Agar Medium)- growth medium
- Plates
- Autoclave
- Anaerobic jar

- Incubator at 30°C

3.1.2 Lactoperoxidase-test at 25°C

The material for this test is identical to the material for the lactoperoxidase-test at 15°C. There are just three differences:

- Fresh raw milk (3 x 100 ml)
- 3 sterilized bottles to keep the raw milk during the test
- Warm water bath at 25°C

3.1.3 Lactoperoxidase-test with the new made H_2O_2 at 15°C

The material for this test is identical to the material for the lactoperoxidase-test at 15°C. There are just a few differences:

- Fresh raw milk (3 x 100 ml)
- 3 sterilized bottles to keep the raw milk during the test
- New made H_2O_2 with new product

This test uses only PCA-plates. MRS-growth medium and M17-growth medium is not necessary.

3.1.4 Lactoperoxidase-test at 15°C with N_2-flushing

The material for this test is identical to the material for the lactoperoxidase-test at 15°C (described in 3.1.1). There are just a few additions:

- There are eight conditions in this test, so 8 x 100 ml raw milk to set up this test.
- Gas bottle that holds N_2-gas (99.999% pure).
- Flow meter to control the flow rate of N_2 to the bottles.

3.1.5 Lactoperoxidase-test at 25°C with N_2-flushing

The material for this test is identical to the material for the lactoperoxidase-test at 15°C with N_2-flushing (described in 3.1.4.).

3.2 Methods

3.2.1 Methods for the lactoperoxidase-test at 15°C

Intent and principle

The intent of this test is to look how effective the lactoperoxidase-system is in different conditions and at different times. In that way conclusions can be made about the effective working and length of lactoperoxidase at 15°C.

Following steps are done to complete this test:

- Making growth media;
- Making substrates;
- Making test bottles and putting up the test;
- Making PCA, MRS and M17-plates with the droplet method;
- Detection;
- Calculating results with described formula.

Making growth-media

Growth-media need to be made following the prescriptions on the packaging of the media.

Each growth-medium detects and proves the presence of different bacteria:

- MRS-medium: detection of *Lactobacillus* spp. (Lab M Ltd, Lancashire, UK)
- M17-medium: detection of *Streptococcus* spp. (Fluka/Sigma-Aldrich, Steinheim, Germany)
- PCA-medium: detection of the total aerobic colony count. (Lab M Ltd, Lancashire, UK)

Substrates

H_2O_2: There has to be made a 30% dilution. (this solution is already in stock) (ref 31642; Riedel de Haën, Seelze,Germany)

Add 8.5 ppm (parts per million) to the milk and 10 ppm (parts per million) of H_2O_2 following FAO. Following calculation was made:

- 1 µl → 1 litre
  = 1 ppm
- 10 ppm
  = 10µl → 1 litre
- Solution at 30 % = 10 µl
  ↓ x 3.3 ↓
  100% or 33.3 µl H_2O_2 for 1 litre
  : 10 ↓
  3.3 µl for 100 ml

In this test, only 75 ml milk is used, so there has to be added 2.48 µl for 75 ml milk.

Thiocyanate: Make a 1% dilution. (ref 251410; Sigma-Aldrich;Germany)

Add 10 ppm (parts per million) to the milk following FAO.
Following calculation is made:

- 1 µl → 1 litre
  = 1 ppm
- 10 ppm
  = 10µl → 1 litre
- Solution at 1,00 % = 10 µl
  ↓ x 100 ↓
  100% or 1000 µl H_2O_2 for 1 litre
  : 10 ↓
  100 µl for 100 ml

In the test, only 75 ml of milk is used, so there has to be added 75 µl of thiocyanate (1%).

<u>The test</u>

Use fresh raw milk in preliminary tests. There will be four test bottles, each with another substrate or additive.

Because of the lack of fresh raw milk, a milk dilution should be made:
220 ml of 48 hours old raw milk at 4°C + 80 ml NaCl (0.85% saline solution)

In this way, it's possible to make four test bottles with each 75 ml of raw milk. The four test bottles are:

- CONTROL: In this bottle, only pure raw milk is present, without any additives.
- HT: Add thiocyanate and hydrogen peroxide (H_2O_2).
- H: Add just hydrogen peroxide (H_2O_2) to the bottle.
- T: Add just thiocyanate to the bottle.

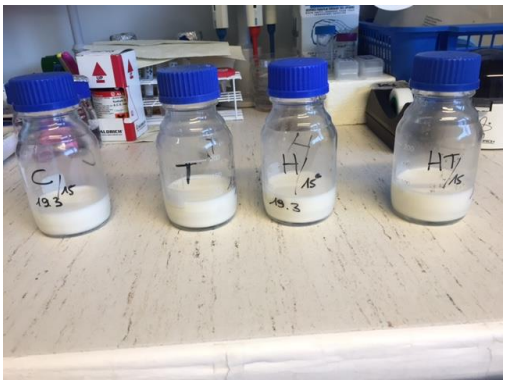


Figure 7: Test bottles

After making the test bottles, they have to be put in a warm water bath at 15°C. Add a magnetic rod in each bottle and place the bottles on the magnetic stirrers in the warm water bath. Attach on each bottle filters to filter the incoming and outgoing air. This has to be done to maintain the sterility of the process. (Munsch-Alatossava *et al.*, 2009)

The bottles stay in the warm water bath during the test period.

Figure 8: Bottles in warm water bath with yellow air filters

Detection

To optimize the effectiveness of the lactoperoxidase-system, by adding the substrates thiocyanate and hydrogen peroxide, the total aerobic microflora, *Streptococcus* spp. and *Lactobacillus* spp. are enumerated during this testing period.

Each bottle is sampled at different times during the testing period:

- t = hours
- t = 24 hours
- t = 48 hours

Various samples can be taken at different hours, for example 2.5 hours or 9 hours after test beginning.

Dilution series in saline water (NaCl 0.85%) are made starting from ….ml of sample. 20 µl of each dilution is plated in twofold or threefold on the three different media by the droplet method.

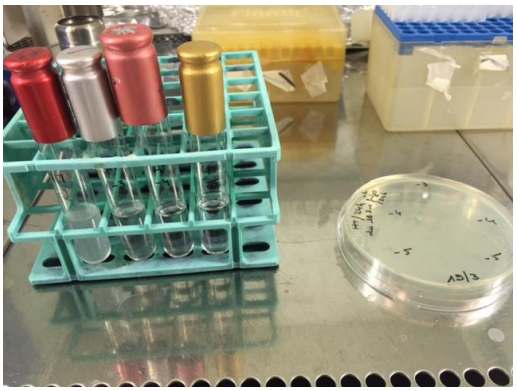


Figure 9: Dilutions

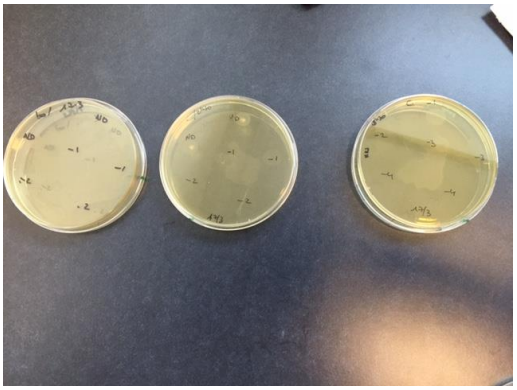


Figure 10: Plates with dilutions on the droplet method

According to IDF, the plates of PCA need to be incubated for 3 days at 30°C. Each day, the plates are read and colony forming units (CFU) are calculated.
The plates of MRS and M17 need to be incubate for 3 days at 30°C in an anaerobic jar. The bacteria to detect here are facultative anaerobe. After three days, plates are read and colony forming units (CFU) are calculated.

Formula

Average count * dilution factor * 50 (20µl on plate of each dilution * 50 = 1ml)
= …. CFU/ml

The log of each colony forming units (CFU) is plotted.

3.2.2 Methods for the lactoperoxidase-test at 25°C

The methods for the test at 25°C are mostly the same as the methods at 15°C. There are just a few little differences. These differences will be written below.

Intent and principle

The intent of this test is to look how effective the lactoperoxidase-system is in different conditions and at different times. In that way conclusions can be made about the effective working and length of lactoperoxidase at 25°C.

The substrates

Same concentrations of the substrates need to be added to the testing bottles following the rules of FAO. But the difference here is that 100 ml of raw milk is added to each bottle. So 3.3 µl of H_2O_2 and 100 µl of thiocyanate must be added at 100 ml of raw milk.

The test

In this test, pure raw milk is used. There are three conditions:

- CONTROL: Only pure raw milk, there are no additives added. This is the reference.
- HT: Add thiocyanate and hydrogen peroxide to the milk.
- H: Add only hydrogen peroxide (H_2O_2) to the milk.

The bottles have to be put in the warm water bath at 25°C for three days.

3.2.3 Methods for the lactoperoxidase-test at 15°C with the new H_2O_2 (hydrogen peroxide)

The methods for this test are identical to the methods described in 3.2.1. There are just a few differences:

- New H_2O_2 is made for performing this test. The H_2O_2 in the other tests written above, was hydrogen peroxide from a few months ago. The H_2O_2 here is brand new.
- There is 100 ml of non-diluted raw milk added to each bottle, so 3.3 µl of H_2O_2 and 100 µl of thiocyanate should be added to 100 ml of raw milk.
- There are just three different conditions: CONTROL, HT and H. In condition HT, both substrates thiocyanate and H_2O_2 are added. In condition H, only H_2O_2 is added.
- Only plates of PCA are made. No MRS-media and M17-media are used.

This test is done to see if there are differences between the effectiveness of the two H_2O_2.

3.2.4 Methods for the lactoperoxidase-test at 15°C with N_2-flushing

The test with N_2-flushing is almost similar to the tests described above. There are just a few differences and additions in this test. The making of growth media and substrates is the same as described in 3.2.1.

Intent and principle

This test is set up to make comparisons between the effectiveness of adding just one or both substrates to activate the lactoperoxidase-system in the raw milk and the effectiveness of N_2-flushing to have an inhibitory effect on the growth of different bacteria, when the raw milk is kept at 15°C.

The test

There are eight conditions in this test:

- CONTROL: only raw milk, without any added substrate or N_2-flushing.
- HT: both substrates are added to the raw milk, thiocyanate and H_2O_2.
- H: only H_2O_2 is added to the raw milk.
- T: only thiocyanate is added to the raw milk.

- CN: N_2-flushing in the CONTROL-bottle.
- HTN: both substrates are added, thiocyanate and H_2O_2, but also N_2-flushing happens.
- HN: H_2O_2 is added and N_2-flushing happens.
- TN: thiocyanate is added and N_2-flushing happens.

There are eight conditions, so 800 ml is needed to make eight bottles with each 100 ml raw milk.

The first conditions: CONTROL, HT, H and T, are the same as described in the experiments above.

The four new conditions: CN, HTN, HN and TN are created following this method:
In each bottle, N_2-gas is introduced in the headspace of the flasks with a constant flow rate of 120 ml/min via 0.2- µm sterile filters. The headspace of the flasks is 130 ml and is continuously purged by fresh N_2-gas. The purged atmosphere is membrane-filtered and removed trough an outlet tube. In this way, gas exchange is possible with the environment. To enable the simultaneous N_2-gas treatment of the several samples, the different flasks are connected to each other in series via tubes holding 0.2- µm filters.

All eight bottles have to be put on a multi-place magnetic stirrer in a warm water bath at 15°C. There is an immersion thermostat to ensure constant temperature, by using ethanol as cooling agent.

Plates of PCA, MRS and M17 are made at different times of each condition. On the one hand to compare differences in effectiveness of the different conditions after more counting points, on the other hand to detect changes in the effectiveness of the different conditions at different hours. So conclusions can be made of the differences between the eight conditions and of the changes at different counting points.

Samples of the bottles should be taken at:
- To = beginning time of the test (=initial count)
- After 24 hours
- After 48 hours

Several samples can be taken at different hours, like 2.5 hours or 9 hours after test beginning.

Detection, dilutions and results are identical to those described in 3.2.1.

3.2.5 Lactoperoxidase-test at 25°C with N_2-flushing

The methods for this test are identical to the methods in the lactoperoxidase-test at 15°C with N_2-flushing (described in 3.2.4). The only difference here is the temperature of the warm water bath. In this test, the raw milk is kept at a temperature of 25°C in the warm water bath.

4 Results

4.1 Results of the lactoperoxidase-system at 15°C

First of all, it's important to make comparatives between the different counting points at different times, but it's also important to examine the quantities found on the different growth media.

4.1.1 Colony forming units on PCA (Plate Count Ager) AFTER 3 DAYS

- Results of the plates made at To (10h30-19/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H	T
	-1/-1	>/>			
	-2/-2	24/26			
	-3/-3	2/1			

Table 3: Results of To at 15°C on PCA

Calculation cfu/ml for CONTROL:

Average count * dilution factor * 50

= (24+26)/2 * (10^2) * 50
= 125 000 cfu/ml
= 5.1 log forming units

In table 3, results of the Colony Forming Units counted in the reference bottle (CONTROL) at To, can be found. This quantity of 5.1 log forming units at To, gives the initial microflora in the raw milk on PCA. This quantity is already high, what means the raw milk is already a few days old and isn't fresh at all.

- Results of the plates made at T1 (18h30-19/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H	T
	-1/-1	>/>	19/15	>/>	>/>
	-2/-2	>/>	NC/2	20/22	>/>
	-3/-3	28/29	NC/NC	NC/NC	28/37

Table 4: Results of T1 at 15°C on PCA

Calculation cfu/ml for CONTROL:

= (28+29)/2 * (10^3) * 50
= 1 425 000 cfu/ml
= 6.2 log forming units

Calculation cfu/ml for HT:

Average count* dilution factor * 50

= (19+15)/2 * (10^1) *50
= 8500 cfu/ml
= 3.9 log forming units

Calculation cfu/ml for H:

Average count * dilution factor * 50

= (20+22)/2 * (10^2) * 50
= 105 000 cfu/ml
= 5.0 log forming units

Calculation cfu/ml for T:

Average count * dilution factor * 50

= (28+37)/2 * (10^3)*50
= 6.2 log forming units

In table 4, results of the Colony Forming Units at T1 can be found, 8 hours after the beginning of the test. The log forming units in the reference bottle (CONTROL) already increased after 8 hours at 15°C. This is normal considering of the higher temperature in which the raw milk is kept. Refrigeration keeps the initial microflora low, higher temperatures like 15°C cause growth of the initial microflora.

When both substrates H_2O_2 and thiocyanate are added to the raw milk, the log forming units are the lowest, even lower than the initial microflora in the CONTROL bottle. So there are less Colony Forming Units counted after 8 hours in the bottle with both substrates, then at To in the CONTROL bottle.

The bottle with only thiocyanate added, isn't effective at all. The growth of the microbiota is already higher than in the bottle with only raw milk.

- Results of the plates made 24 hours after To (10h30-20/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H	T
	-1/-1		>/>		
	-2/-2		43/>		
	-3/-3		6/13	>/>	
	-4/-4	>/>		>/>	>/>
	-5/-5	>/>		16/33	>/>
	-6/-6	8/17			16/12

Table 5: Results after 24 hours at 15°C on PCA

Calculation cfu/ml for CONTROL:

= (8+17)/2 * (10^6) * 50
= 625 000 000 cfu/ml
= 8.8 log forming units

Calculation cfu/ml for HT:

Average count* dilution factor * 50

= (6+13)/2 * (10^3) *50
= 475 000 cfu/ml
= 5.7 log forming units

Calculation cfu/ml for H:

Average count * dilution factor * 50

= (16+33)/2 * (10^5) * 50
= 8.1 log forming units

Calculation cfu/ml for T:

Average count * dilution factor * 50

= (16+12)/2 * (10^6)*50
= 8.8 log forming units

In table 5, results can be found of the Colony Forming Units present in each test bottle 24 hours after To. The log forming units in the CONTROL bottle keep growing, until higher quantities. There is an increase of about 2 log forming units, which is a big difference.

The log forming units in the bottle with both substrates thiocyanate and H_2O_2 added, still have the lowest quantities, which is positive. But there is an increase of about 2 log forming units, so the effectiveness decreases.

As told at T1, here after 24 hours, the log forming units in the bottle with only thiocyanate are similar to the Colony Forming Units found in de reference bottle. So adding only thiocyanate or not makes no difference.

The bottle with only H_2O_2 added, also shows a lot of bacteria. There are not lots of differences between the quantities found here, and the quantities found in the bottles with CONTROL and T.

- Results of the plates made 48 hours after To (10h30-21/3/2015)

AFTER 3 DAYS	**Dilution**	**CONTROL**	**HT**	**H**	**T**
	-4/-4		>/>		
	-5/-5	>/>	>/>	>/>	>/>
	-6/-6	38/40	24/21	32/40	24/32
	-7/-7	5/4		5/3	6/7

Table 6: Results after 48 hours at 15°C on PCA

Calculation cfu/ml for CONTROL:

= (38+40)/2 * (10^6) * 50
= 195 000 000 cfu/ml
= 8.3 log forming units

Calculation cfu/ml for HT:

Average count* dilution factor * 50

= (24+21)/2 * (10^6) *50
= 9.1 log forming units

Calculation cfu/ml for H:

Average count * dilution factor * 50

= (32+40)/2 * (10^6) * 50
= 9.2 log forming units

Calculation cfu/ml for T:

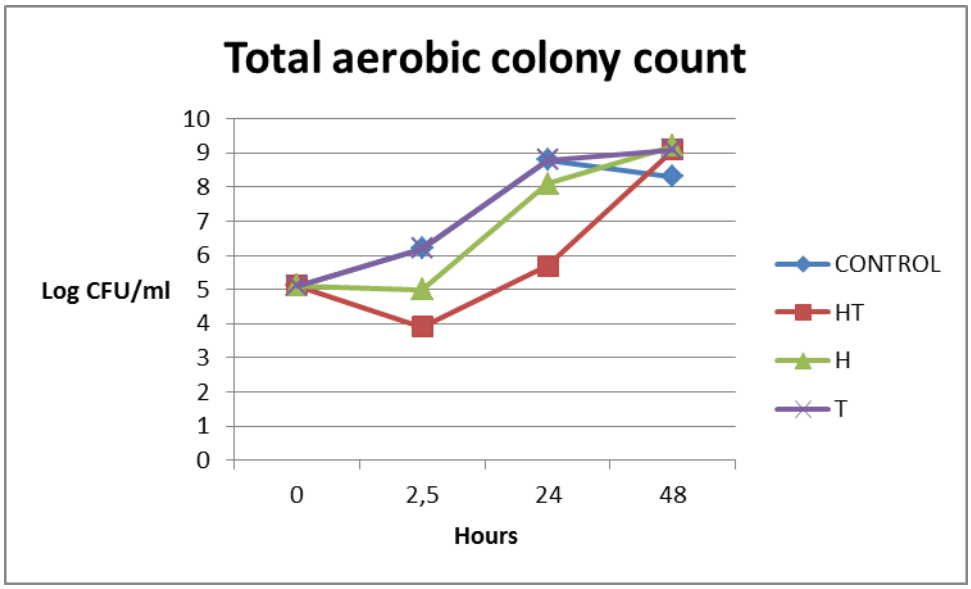
Average count * dilution factor * 50

= (24+32)/2 * (10^6)*50
= 9.1 log forming units

In table 6, results of the Colony Forming Units can be found, 48 hours after To. Calculations above show that growth of the bacteria is a fact. Growth of the initial microflora is lowest in the bottle with only raw milk added. This is a rather strange result.

In the bottles where both substrates are added, same quantities are counted. There is no lower growth in the bottle with both thiocyanate and H_2O_2. The substrates don't work anymore after 48 hours at 15°C.

- Graphic



Graphic 1: Total aerobic colony count at 15°C

In this graphic, the different quantities are shown of the total aerobic colony counts at different moments and in the different test bottles. The initial microflora is the same for each situation. The results 8 hours after To show some interesting things. The Colony Forming Units counted in the CONTROL-bottle and the bottle with only thiocyanate added, are the same. This means adding only thiocyanate isn't useful. When only thiocyanate is added, it doesn't activate the lactoperoxidase system, even after 24 hours and 48 hours, the CFU's are the same as in the CONTROL-bottle, they are even higher. So in this case, there is no bacteriostatic or bactericidal effect of the lactoperoxidase-system.

The bottle with both thiocyanate and H_2O_2 added, seems most efficiently. 8 hours after To, there is a decrease of the initial microflora, which means that the lactoperoxidase system is activated and has a bactericidal effect on the total aerobic colony count. This is a positive result. After 24 hours, the growth of the total aerobic colony count increased with two log forming units, there is growth but lower than in the other test bottles. So there is a retarded growth. This system is even effective after 24 hours, because of the lower growth of the bacteria. Here there are 5.7 log forming units found, in the other bottles, the log forming units found were between 8.1 and 8.8.

After 48 hours however, there are no more differences found. The results of the CFU's in each bottle are almost the same, the growth also increased a lot in the bottle with both thiocyanate and H_2O_2. So the activation of the lactoperoxidase-system stopped. That can also be read in the literature study. The effectiveness of the lactoperoxidase-system at 15°C stays for 24-26 hours.

When examining the results of the bottle with only H_2O_2, growth decreased a little bit after 8 hours. But even after 24 hours, the substrate hasn't any effect on the activation of the lactoperoxidase system. Just adding H_2O_2, isn't useful to activate the lactoperoxidase-system and to have a bacteriostatic or bactericidal effect on the total aerobic colony count.

4.1.2 Colony forming units on MRS AFTER 3 DAYS

- Results of the plates made at To (10h30-19/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H	T
	-1/-1	NC/NC			
	-2/-2	NC/NC			
	-3/-3	NC/NC			

Table 7: Results of To at 15°C on MRS-medium

In table 7, there are no Colony Forming Units found in the CONTROL-bottle. It's not possible to say there is no initial microflora of *Lactobacillus* spp., there are just no counts. Further results will be examined.

- Results of the plates made 48h after To (10h30-21/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H	T
	-1/-1	>	>	>	>
	-2/-2	>	35	>	>
	-3/-3	>	3/5	>	>
	-4/-4	26/17	1	16	20/26
	-5/-5		NC	1	3

	-6/-6			0	0/2

Table 8: Results after 48 hours at 15°C on MRS-medium

Calculation cfu/ml for CONTROL:

= (26+17)/2 * (10^4) * 50
= 7.0 log forming units

Calculation cfu/ml for HT:

Average count* dilution factor * 50

= (3+5)/2 * (10^3) *50
= 5.3 log forming units

Calculation cfu/ml for H:

Average count * dilution factor * 50

= 16 * (10^4) * 50
= 6.9 log forming units

Calculation cfu/ml for T:

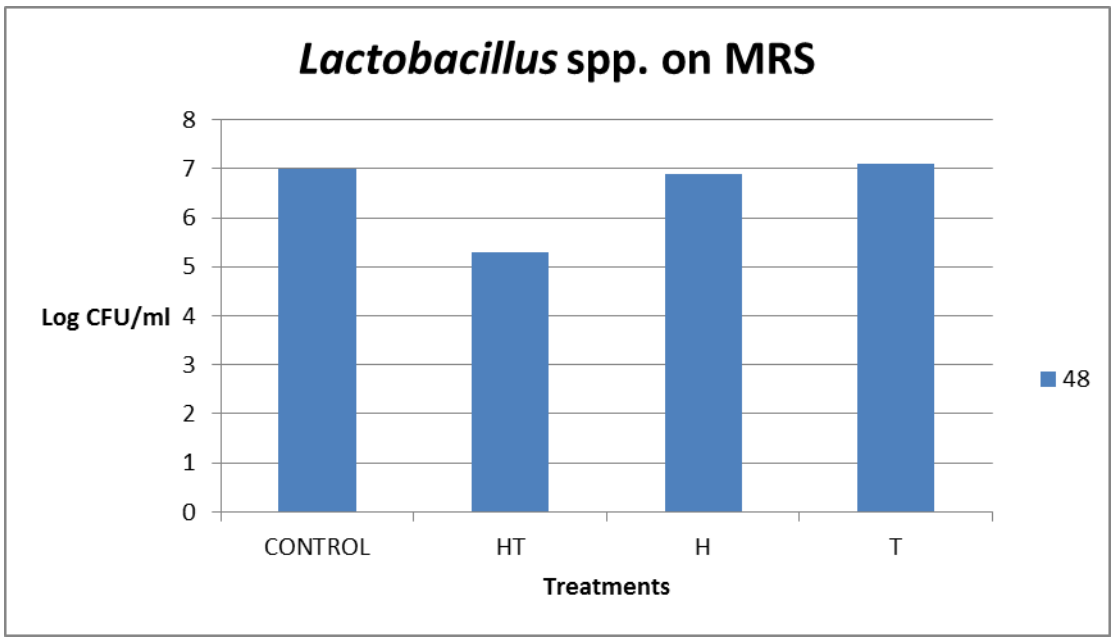
Average count * dilution factor * 50

= (20+26)/2 * (10^4)*50
= 7.1 log forming units

In table 8, results can be found of the plates made 48 hours after To on the MRS-medium. The growth of *Lactobacillus* spp. in the CONTROL-bottle increased a lot, from no countable counts to 7.0 log forming units. Here on MRS, there are also similarities between the Colony Forming Units in the CONTROL-bottle and the bottle with only thiocyanate. The growth of *Lactobacillus* spp. is even higher than in the CONTROL bottle.

The log forming units are the lowest in the bottle with both thiocyanate and H_2O_2. The bottle with only H_2O_2 added, shows results between the two extremes.

- Graphic



Graphic 2: *Lactobacillus* spp. at 15°C

When taking a look at the effect of the lactoperoxidase-system on the growth of *Lactobacillus* spp., there are only results after 48 hours. There, the same conclusions and objections can be made as on PCA. Only adding thiocyanate to the raw milk, has no effect because of the highest growth. The growth is even higher than in the bottle with just raw milk. Adding both thiocyanate and H_2O_2 is still the most effective, even after 48 hours, the lactoperoxidase-system has the best working and shows the lowest quantity of *Lactobacillus* spp. The bottle with only H_2O_2 added, isn't effective at all. It shows the same results as the CONTROL and T.

4.1.3 Colony forming units on M17 AFTER 3 DAYS

- Results of the plates made 48 hours after To (10h30-21/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H	T
	-1/-1		>	>	>
	-2/-2		30	>	>
	-3/-3	>	5	>	>
	-4/-4	22/21	NC	14	23
	-5/-5	0/2	NC	5	4
	-6/-6	0/1	NC	1	NC

Table 9: Results 48 hours after To at 15°C on M17

Calculation cfu/ml for CONTROL:

= (22+21)/2 * (10^4) * 50
= 7.0 log forming units

Calculation cfu/ml for HT:

Average count* dilution factor * 50

= 30 * (10^2) *50
= 5.2 log forming units

Calculation cfu/ml for H:

Average count * dilution factor * 50

= 14 * (10^4) * 50
= 6.8 log forming units

Calculation cfu/ml for T:

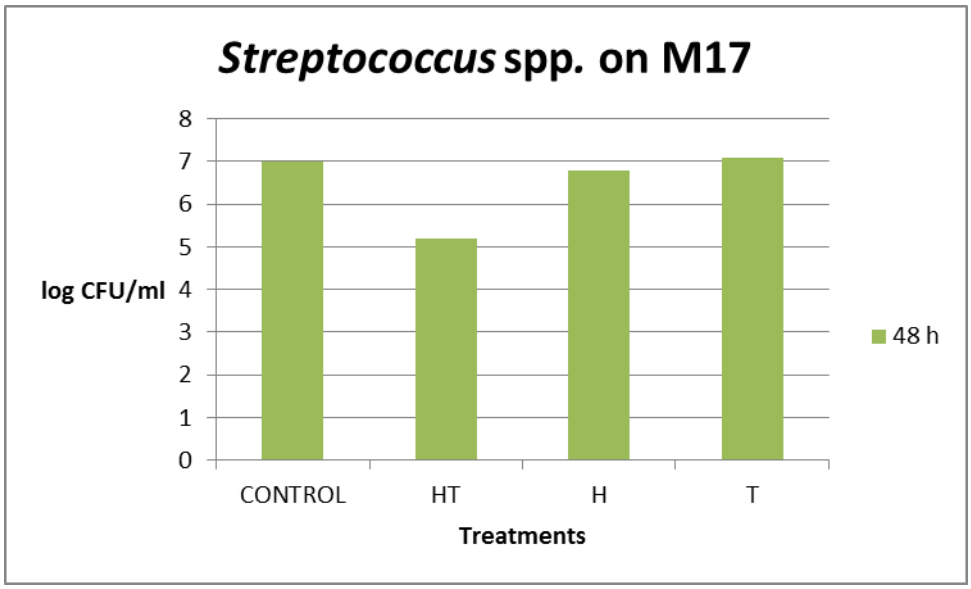
Average count * dilution factor * 50

= 23 * (10^4)*50
= 7.1 log forming units

For the Colony Forming Units on M17, there is only one counting point noted. That's the point 48 hours after To. The growth of *Streptococcus* spp. here is still high after 48 hours. The quantities on M17 for the four test bottles have the same comparative results as on PCA and MRS. The log forming units of the CONTROL-bottle and the bottle with thiocyanate are almost the same, the bottle with only thiocyanate has even higher quantities than the CONTROL-bottle. The bottle with only H_2O_2 has also high quantities of growth, that's almost the same as the other two, described above.

The bottle with both thiocyanate and H_2O_2 added, also has high quantities of the Colony Forming Units, but the results are still the lowest.

- Graphic



Graphic 3: *Streptococcus* spp. at 15°C

The explanation here is the same as written above at the point 48 hours after To.
For the growth of *Streptococcus* spp., there is no initial microflora count. There are only results after 48 hours. The results for the four conditions are the same as described on MRS, so the same conclusion can be made.

4.2 Results of the lactoperoxidase-system at 25°C

In the discussion of the lactoperoxidase-test at 25°C, the results of each growth medium taken at different times are examined. After that the differences between the results on the different growth media will be explained.

4.2.1 Colony forming units on PCA (Plate Count Agar) AFTER 3 DAYS

- Results of the plates made at To (17/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H
	ND/ND	>/>		
	-1/-1	17/17		
	-2/-2	4/3		

Table 10: Results of To at 25°C on PCA

Calculation cfu/ml for CONTROL:

Average count * dilution factor * 50

= (17+17)/2 * (10^1) * 50
= 3.9 log forming units

In table 10, the results of the initial microflora in the CONTROL-bottle can be found. This quantity will be kept as reference. While looking at the results on PCA-medium, there is an initial microflora of 3.9 log forming units. The quantity of the total aerobic colony count at To in this test is a lot lower than in the lactoperoxidase-test at 15°C so the raw milk here is fresher. But there is still a certain population of microflora present.

- Results of the plates made at T1 (after 2h30-17/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H
	ND/ND/ND	62/>	>/>	49/51
	-1/-1	22/18	12/11	23/22
	-2/-2	2/3	3/2	3/3

Table 11: Results of T1 at 25°C on PCA

Calculation cfu/ml for CONTROL:

= (22+18)/2 * (10^1) * 50
= 4.0 log forming units

Calculation cfu/ml for HT:

Average count* dilution factor * 50

= (12+11)/2 * (10^1) *50
= 3.8 log forming units

Calculation cfu/ml for H:

Average count * dilution factor * 50

= (23+22)/2 * (10^1) * 50
= 4.1 log forming units

In table 11, results of the three conditions CONTROL, HT and H can be found. After 2.5 hours, there are not a lot of changes. The little differences between the quantities at To and T1, may be explained by the time between the two counting points. There are just 2.5 hours between, that's little. At T1 (2.5 hours after To), the growth of the initial microflora increased a little bit in the CONTROL-bottle and in the bottle with only H_2O_2. The increase is not so high, but there are just 2.5 hours between To and T1, so that can be the explanation. In the bottle with both substrates (thiocyanate and H_2O_2), the initial microflora decreased a little bit. Not so much but there is a decrease after 2.5 hours, so the lactoperoxidase-system is activated by the substrates and has a bacteriostatic and a little bactericidal effect on the total aerobic colony count. Condition HT keeps the bacterial growth the lowest. The CONTROL-bottle and the bottle with just H_2O_2, have higher values. This H-bottle, has the least effect.

- Results of the plates made at T2 (18h30-17/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H
	-1/-1	>	12/14	71/78
	-2/-2	>	2/3	35/35
	-3/-3	55/56	2/NC	8/5
	-4/-4	10/9		

Table 12: Results of T2 at 25°C on PCA

Calculation cfu/ml for CONTROL:

= (10+9)/2 * (10^4) * 50
= 6.7 log forming units

Calculation cfu/ml for HT:

Average count* dilution factor * 50

= (12+14)/2 * (10^1) *50
= 3.8 log forming units

Calculation cfu/ml for H:

Average count * dilution factor * 50

= (8+5)/2 * (10^3) * 50
= 5.5 log forming units

In table 12, the results can be found at T2, 9 hours after the first counting point (To). An increase can be seen of the CFU's in the CONTROL-bottle and the bottle with H_2O_2. Growth in the bottle with only raw milk, is the highest, no substrates are added so there is no activation of the lactoperoxidase-system. Under the condition of 25°C, the initial microflora keeps increasing all the time. So holding raw milk at 25°C isn't good at all for preserving raw milk and keeping the initial microflora low. There is an increase of the CFU's in bottle H, but

the growth isn't as high as in the CONTROL, which is also positive. So there can be said that the lactoperoxidase-system is still active in the bottle with only H_2O_2 added. The growth is not as high as in the CONTROL-bottle, which means that there is delayed growth, so the lactoperoxidase-system is activated but not enough to have a bacteriostatic or bactericidal effect on the microflora in this condition.
After 9 hours, the microbial count in the bottle with both thiocyanate and H_2O_2, stays the same as after two hours and has a bacteriostatic effect on the total aerobic colony count. Adding both thiocyanate and H_2O_2 has a positive effect up to 9 hours after To. This is a positive result. The lactoperoxidase-system is activated.

- Results of the plates made 24 hours after To (18/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H
	ND/ND		>/>	
	-1/-1		>/>	
	-2/-2		>/>	
	-3/-3		>	>/>
	-4/-4	>/>	82/65	>/>
	-5/-5	>/>	8/9	38
	-6/-6	14/15		4

Table 13: Results 24 hours after To at 25°C on PCA

Calculation cfu/ml for CONTROL:

= (14+15)/2 * (10^6) * 50
= 8.9 log forming units

Calculation cfu/ml for HT:

Average count* dilution factor * 50

= (8+9)/2 * (10^5) *50
= 7.6 log forming units

Calculation cfu/ml for H:

Average count * dilution factor * 50

= (4)* (10^6) * 50
= 8.3 log forming units

Looking at the results in table 13, 24 hours after To, some interesting facts appear. There is a high increase of the total aerobic colony count in each condition. The CFU's in the bottle with both thiocyanate and H_2O_2, stay the lowest but are still high enough to say that the lactoperoxidase-system stopped working. 24 hours after To, the lactoperoxidase-system doesn't work anymore at 25°C.

The CFU's in the CONTROL-bottle are the highest.

- Results of the plates made 48 hours after To (19/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H
	-4/-4	>/>	>/>	>/>
	-5/-5	>/>	>/>	>/>
	-6/-6	35/21	47/57	23/29

Table 14: Results 48 hours after To at 25°C on PCA

Calculation cfu/ml for CONTROL:

= (35+21)/2 * (10^6) * 50
= 9.1 log forming units

Calculation cfu/ml for HT:

Average count* dilution factor * 50

= (47+57)/2 * (10^5) *50
= 9.4 log forming units

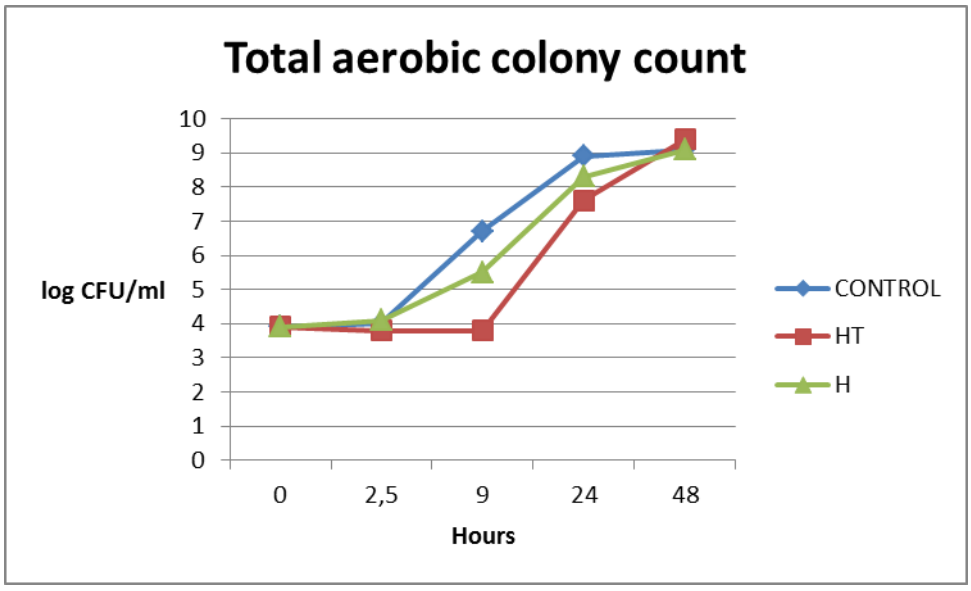
Calculation cfu/ml for H:

Average count * dilution factor * 50

= (23+29)* (10^6) * 50
= 9.1 log forming units

The results shown here, prove the increasing growth of the total aerobic colony count. The quantities of the bacteria in each bottle are the same, there are almost no differences. So the substrates added, have no more effect on the lactoperoxidase-system. The initial microflora keeps growing.

- Graphic



Graphic 4: Total aerobic colony count at 25°C

As mentioned above, the bottle with both thiocyanate and H_2O_2 added, is the most efficient and causes the activation of the lactoperoxidase-system in a period of 9 hours after To. That's also written in the literature study. The lactoperoxidase-system has an effect until 11-12 hours after To at 25°C. After 24 and 48 hours there are no more differences between the quantities in the different conditions, so the lactoperoxidase-system doesn't work anymore in any condition. The growth in the HT-bottle stays the lowest, but an increasing growth can be perceived after 9 hours.

So, the lactoperoxidase-system is best activated when both substrates are added and has an influence on the total aerobic colony count on PCA up to 9 hours after To.

4.2.2 Colony forming units on MRS AFTER 3 DAYS

- Results of the plates made at To (17/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H
	ND/ND	NC/NC		
	-1/-1	NC/NC		
	-2/-2	NC/NC		

Table 15: Results of To at 25°C on MRS

There are no colony forming units detected in the CONTROL-bottle. On MRS, there is no initial microflora count at To. So there is no *Lactobacillus* spp. present in the raw milk.

- Results of the plates made at T1 (after 2h30-17/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H
	ND/ND	NC/NC	NC/NC	2/NC
	-1/-1	NC/NC	NC/NC	NC/NC
	-2/-2	NC/NC	NC/NC	NC/NC
	-3/-3	NC/NC		

Table 16: Results of T1 at 25°C on MRS

Calculation cfu/ml for CONTROL:

No colony forming units found in the CONTROL.

Calculation cfu/ml for HT:

No colony forming units found in the HT.

Calculation cfu/ml for H:

Average count * dilution factor * 50

= (2+0)/2 * 50
= 1.7 log forming units

While examining the results in table 16, there are no counts of colonies of *Lactobacillus* spp. in the bottles with CONTROL and HT, so there is no growth at all. That's normal because of

the absence of initial microflora at To. In the bottle with H_2O_2, there can be found a little growth of *Lactobacillus* spp. But because of the very little growth compared to the results in CONTROL and HT, there can be said that this is contamination.

- Results of the plates made at T2 (18h30-17/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H
	-1/-1	NC/NC	NC/NC	NC/NC
	-2/-2	NC/NC	NC/NC	NC/NC
	-3/-3	NC/NC	NC/NC	NC/NC
	-4/-4	NC/NC	NC/NC	NC/NC

Table 17: Result of T2 at 25°C on MRS

At T2(9 hours after To), there is no growth of *Lactobacillus* spp. in the bottles with three different conditions. So this proves the explanation of the contamination at T1.

- Results of the plates made 24 hours after To (18/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H
	-1/-1	21	NC	3/4
	-2/-2	2	NC	NC
	-3/-3	NC		NC
	-4/-4	NC		
	-5/-5	NC		
	-6/-6	NC		

Table 18: Results 24 hours after To at 25°C on MRS

Calculation cfu/ml for CONTROL:

= 21 * (10^1) * 50
= 4.0 log forming units

Calculation cfu/ml for HT:

No colony forming units in HT.

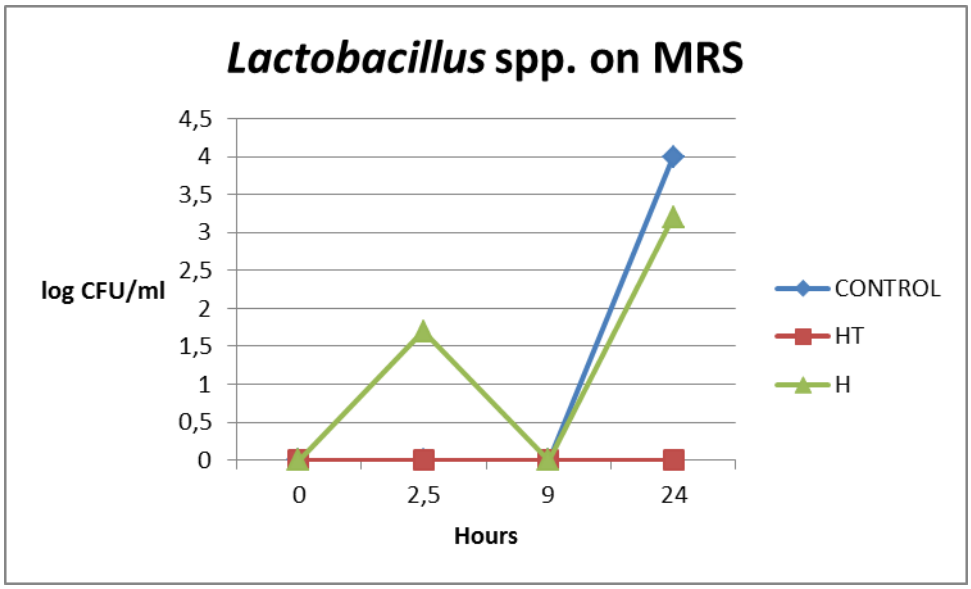
Calculation cfu/ml for H:

Average count * dilution factor * 50

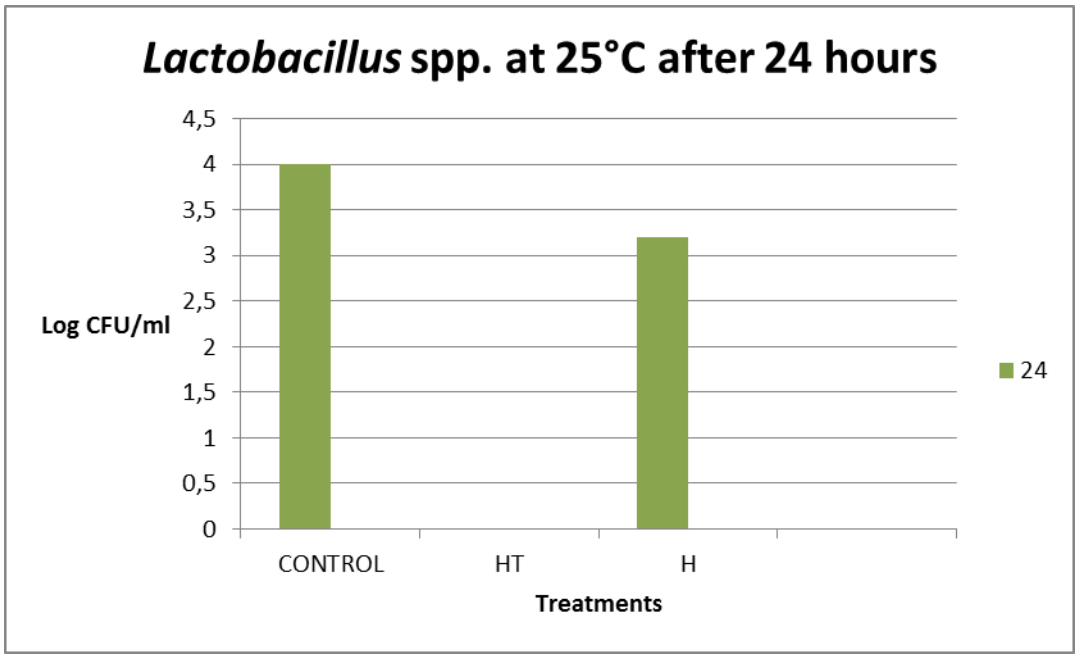
= (3+4)/2* (10^1) * 50
= 3.2 log forming units

After 24 hours, there is a little growth of *Lactobacillus* spp. on MRS-medium. How this is possible, because of the no counting colonies in the previous counting points, can't be explained. Maybe this is contamination.

- Graphic



Graphic 5: *Lactobacillus* spp. at 25°C



Graphic 6: *Lactobacillus* spp. at 25°C after 24 hours

The graphic of the CFU's of *Lactobacillus* spp. on MRS, is shown above. The graphic shows strange results. Most of the counting points, show no CFU's. So there is no growth of *Lactobacillus* spp. But after 24 hours, there is a little count in the CONTROL-bottle and the bottle with just H. Also after 2.5 hours, there is a little count in the bottle of H. This is probably contamination.

Comparison between the results on PCA and MRS can't be made because of the results of MRS, where no plausible results were found.

4.2.3 Colony forming units on M17 AFTER 3 DAYS

- Results of the plates made at To (17/3/2015)

AFTER 3 DAYS	Dilution	CONTROL	HT	H
	ND/ND	32/40		
	-1/-1	2/10		
	-2/-2	1/NC		

Table 19: Results of To at 25°C on M17

Calculation cfu/ml for CONTROL:

= (2+10)/2 * (10^1) * 50
= 3.5 log forming units

The initial microflora of *Streptococcus* spp. in the CONTROL-bottle is 3.5 log forming units. This is the reference. On M17-medium, there are only results for the CONTROL-bottle. No plates were made for the bottles with HT and H. So the evolution of the growth of *Streptococcus* spp. can be followed for raw milk kept at 25°C.

- Results of the plates made at T1 (after 2h30-17/3/2015)

AFTER 3 DAYS	Dilution	CONTROL
	ND/ND	48/38
	-1/-1	5/4
	-2/-2	NC/NC
	-3/-3	

Table 20: Results of T1 at 25°C on M17

Calculation cfu/ml for CONTROL:

Average count * dilution factor * 50

= (5+4)/2 * (10^1) * 50
= 3.4 log forming units

There is no growth of *Streptococcus* spp. in the CONTROL-bottle after 2.5 hours. The initial microflora stays stable.

- Results of the plates made 24 hours after To (18/3/2015)

AFTER 3 DAYS	Dilution	CONTROL
	-1/-1	>
	-2/-2	>
	-3/-3	>
	-4/-4	>
	-5/-5	>
	-6/-6	12

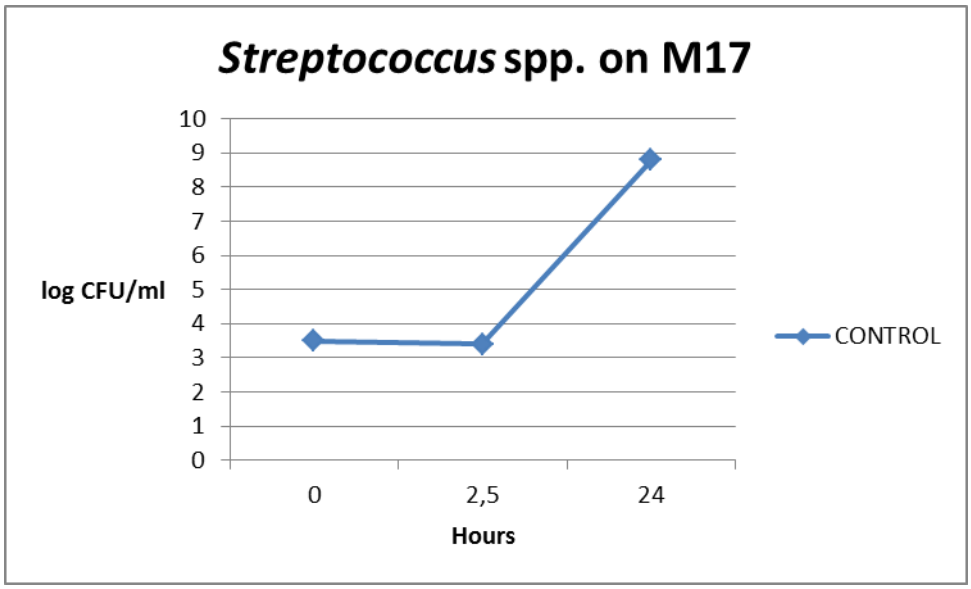
Table 21: Results 24 hours after T1 at 25°C on M17

Calculation cfu/ml for CONTROL:

= 12 * (10^6) * 50
= 8.8 log forming units

The results of table 21, show the increase of the growth of *Streptococcus* spp. on M17-medium 24 hours after To. There is a big increase of the growth. So keeping raw milk at 25°C, after milking, isn't a possibility at all. *Streptococcus* spp. keep growing. The effect of the substrates on the lactoperoxidase-system can't be controlled here because there were no plates made of these conditions.

- Graphic



Graphic 7: *Streptococcus* spp. at 25°C

The test on M17 only shows results of the counts of *Streptococcus* spp. in the bottle with raw milk. So the effectiveness and working of the lactoperoxidase-system can't be proved for this microorganism because of the lack of activation by the substrates thiocyanate or H_2O_2. First, *Streptococcus* spp. is present in the raw milk, and growth stays stable until 2.5 hours after To. After 24 hours, the growth of this microorganism increased a lot to 8.8 log forming units.

4.3 Results of the lactoperoxidase-system with the new H_2O_2 at 15°C

Comparisons were made on PCA-medium at three different times: at To, after two hours and after 24 hours.

4.3.1 Colony forming units on PCA (Plate Count Ager)

- Results of the plates made at To (10h30-25/3/2015)

	Dilution	CONTROL	HT	H(new)T
DAY 1	-1/-1	>/>		
	-2/-2	30/23		
	-3/-3	4/5		
DAY 2	-1/-1	>/>		
	-2/-2	33/23		
	-3/-3	5/5		

Table 22: Results of To at 15°C on PCA

Calculation cfu/ml for CONTROL:

Average count * dilution factor * 50

= (33+23)/2 * 10^2 * 50
= 5.1 log forming units

In table 22, the count is shown of the initial microflora in the sample of the raw milk. This is the reference. The quantity of total aerobic colony count is high. At To, there are 5.1 log forming units found in the CONTROL-bottle. The reason here is the freshness of the raw milk. This raw milk was kept a few days in the refrigerator at 4°C. So the raw milk isn't that fresh. In this test, the difference between the working of the old H_2O_2 and the new made H_2O_2 on the lactoperoxidase-system is tested at 15°C.

- Results of the plates made at T1 (12u30-25/3/2015)

	Dilution	CONTROL	HT	H(new)T
DAY 1	-1/-1		8/14	13/15
	-2/-2	27	1/NC	2/1
	-3/-3	2	1/NC	NC/NC
	-4/-4	NC/NC		
DAY 2	-1/-1		25/27	25/18
	-2/-2	27	3/3	4/7
	-3/-3	5/2	1/2	NC/NC
	-4/-4	NC/NC		

Table 23: Results of T1 at 15°C on PCA

Calculation cfu/ml for CONTROL:

= 27 * 10^2 * 50
= 5.1 log forming units

Calculation cfu/ml for HT:

Average count* dilution factor * 50

= (25+27)/2 * (10^1) *50
= 4.1 log forming units

Calculation cfu/ml for H(new)T:

Average count * dilution factor * 50

= (25+18)/2 * (10^1) * 50
= 4.0 log forming units

The results of the plates made 2 hours after To are written in table 23. The CFU's are still the same in the CONTROL-bottle after 2 hours. The time between To and T1 isn't big at all, there are just two hours between To and T1, so that could be the explanation. When examining the results of the bottles with the old H_2O_2 and the new H_2O_2, that gives us the same results. So there is no difference between the effectiveness of the lactoperoxidase-system with the old H_2O_2 and the new H_2O_2. In both conditions, the lactoperoxidase-system is working. It has a bactericidal effect on the total aerobic colony count, because of the decrease in CFU's from 5.1 log forming units (To) to 4.0-4.1 log forming units (T1).

- Results of the plates made 24 hours after To (10u30-26/3/2015) AFTER 3 DAYS

AFTER 3 DAYS	Dilution	CONTROL	HT	H(new)T
	-1/-1		20/11	14/11
	-2/-2		3/3	1/2
	-3/-3	>/>	1/NC	NC/NC
	-4/-4	>/>		
	-5/-5	27/34		

Table 24: Result 24 hours after To at 15°C on PCA

Calculation cfu/ml for CONTROL:

= (27+34)/2 * (10^5) * 50
= 8.2 log forming units

Calculation cfu/ml for HT:

Average count* dilution factor * 50

= (20+11)/2 * (10^1) *50
= 3.9 log forming units

Calculation cfu/ml for H(new)T:

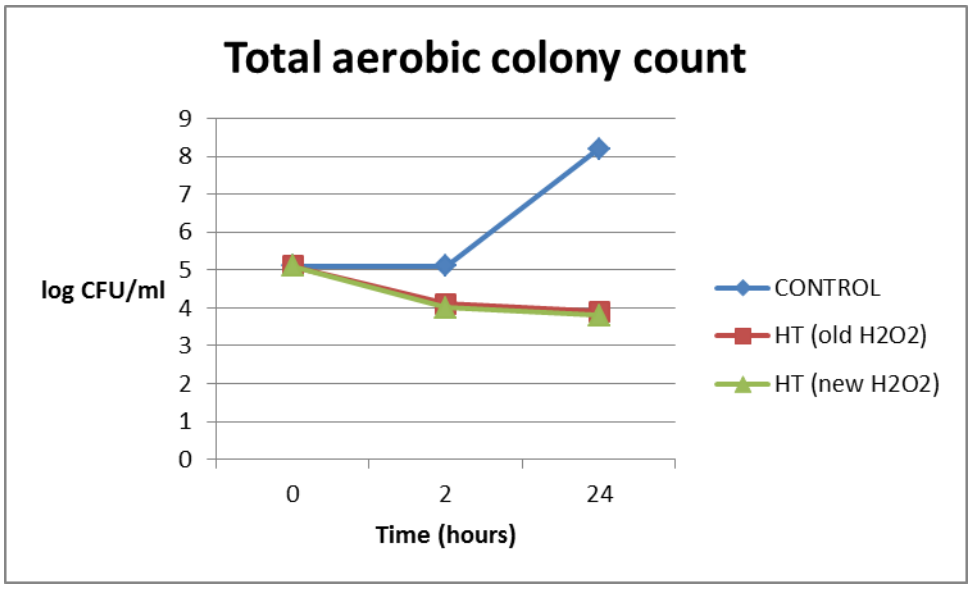
Average count * dilution factor * 50

= (14+11)/2 *(10^1) * 50
= 3.8 log forming units

After 24 hours, there is an increase of the log forming units in the raw milk (CONTROL). This is quite normal, because of the temperature in which the raw milk is kept. The results of the counts of the samples with the old and the new H_2O_2 are almost identical. There is just a little decrease of the log forming units, so the lactoperoxidase-system stays active after 24 hours

and has a bacteriostatic and a little bactericidal effect on the total aerobic colony count in both situations. That's also what's written in the literature study. The lactoperoxidase-system is activated up to 24-26 hours after To at 15°C, when both substrates (thiocyanate and H_2O_2) are added.

- Graphic



Graphic 8: Total aerobic colony count at 15°C with the new H_2O_2

As described above, there is no difference between the influence of the old or new H_2O_2 on the effectiveness of the lactoperoxidase-system. The substrates cause a decrease in the total aerobic colony count. The CFU's in the CONTROL-bottle stay increasing. So, there can be concluded that the new made H_2O_2 has the identical effect on the lactoperoxidase-system as the old H_2O_2.

4.4 Results of the first lactoperoxidase-test at 15°C with N_2-flushing

There are eight conditions in this test:

- CONTROL: only raw milk, without any substrate or N_2-flushing added.
- HT: both substrates are added to the raw milk, thiocyanate and H_2O_2.
- H: only H_2O_2 is added to the raw milk.
- T: only thiocyanate is added to the raw milk.
- CN: N_2-flushing in the CONTROL-bottle.
- HTN: both substrates are added, thiocyanate and H_2O_2, but also N_2-flushing happens.
- HN: H_2O_2 is added and N_2-flushing happens.
- TN: thiocyanate is added and N_2-flushing happens.

In this test, differences between the eight conditions are examined and compared. Tests are done on three media: PCA, MRS and M17.

4.4.1 Colony forming units on PCA (Plate Count Agar)

- Results of the plates made at To (9h15 – 23/3/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	ND/ND/ND	>/>/>							
	-1/-1/-1	9/6/8							
	-2/-2								
DAY 2	ND/ND/ND	>/>/>							
	-1/-1/-1	18/7/23							
DAY 3	ND/ND/ND	>/>/>							
	-1/-1/-1	24/19/25							

Table 25: Results of To at 15°C with N_2-flushing

Log forming units of the plates after 3 days

The quantities found in the table written below, are the results of following calculations:

Average count * dilution factor * 50

= result → of this result, the log is taken

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	4.1							

Table 26: Log forming units at To on PCA with N_2-flushing

When looking on PCA, the initial microflora of the total aerobic colony count is moderate. The results found in table 25 and 26, are the Colony Forming Units on PCA at To in the

CONTROL-bottle. There are 4.1 log forming units found at To. This is a moderate value, which means that the raw milk isn't very fresh. The milk is already a few days old or the milk comes from different farms. But the value is acceptable.

- Results of the plates made at T1 (11h45-23/3/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	ND	59	24	38	>	>	22	>	>
	-1/-1/-1	10/10/5	4/9/5	11/10/18	15/9/10	10/9/11	3/3/3	12/10/9	10/16/10
	-2/-2/-2	3/1/ NC	2/2/NC	2/1/NC	1/1/NC	1/3/NC	NC/1/1	NC/NC/1	2/3/2
DAY 2	ND	>	>	>	>	>	>	>	>
	-1/-1/-1	16/13/13	13/15/19	24/15/20	26/13/17	24/16/17	>/3/10	17/15/12	22/26/15
	-2/-2/-2	8/4/3	3/3/NC	3/4/2	3/3/NC	3/5/1	NC/1/1	NC/1/1	4/4/2
DAY 3	ND	>	>	>	>	>	>	>	>
	-1/-1/-1	19/16/16	14/17/21	25/19/23	26/13/18	24/17/17	>/15/17	23/21/12	24/27/19
	-2/-2/-2	8/5/3	4/4/NC	3/4/2	3/3/NC	3/5/2	1/1/1	NC/2/1	4/5/2

Table 27: Results of T1 at 15°C on PCA with N_2-flushing

Log forming units of the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.9	3.9	4.0	4.0	4.0	3.9	4.0	4.1

Table 28: Log forming units at T1 with N_2-flushing

2h30 after To (=T1), results don't change in high quantities. There are just 2.5 hours between the two counting points. This may be the explanation here. In the control bottle there is a decrease in counts. This is strange, because the control bottle doesn't contain any substrate or N_2-flushing. So activation of the lactoperoxidase-system isn't possible. The reason for this could be inaccuracy during the experiment. But differences in quantities are very low. In the bottle where both thiocyanate and H_2O_2 are added, there is also a light decrease of counted quantities. This means activation of the lactoperoxidase-system by both substrates. In this case, the lactoperoxidase-system has a bactericidal effect on the total aerobic colony count after 2h30 at 15°C. In the bottles with only thiocyanate or H_2O_2, there is even a decrease in log forming units, which means the activation of the lactoperoxidase-system with a bactericidal effect.

In the bottle with N_2-flushing, without addition of any substrate, there is a light decrease of the total aerobic colony count. This means that N_2-flushing has a bactericidal effect on the total aerobic colony count after 2h30 at 15°C. In this case, substrates aren't necessary because of the effect of N_2-flushing. Also the bottle with both substrates and N_2-flushing shows the same results, even as the bottle whit H_2O_2 added and N_2-flushing. The bottle with addition of thiocyanate and N_2-flushing, shows the same result of colony forming units as at To. This means that the combination of thiocyanate and N_2-flushing, has an bacteriostatic effect on the total aerobic count after 2h30. The initial bacteria can't grow, but they're not killed at either. This condition is the least efficient of the eight different conditions.

- Results of the plates made at T2 (18h30-23/3/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	ND/ND/ND		NC/NC/NC	NC/NC/NC		>/>	NC/NC/NC	>/>/>	>/>/>
	-1/-1/-1	3/3/4	NC/NC/NC	NC/NC/NC	NC/2/5	4/8/10	NC/NC/NC	9/5/5	1/12/13
	-2/-2/-2	3/2/NC		NC/NC/NC	1/1/NC	NC		2	NC
DAY 2	ND/ND/ND		>/>/>	>/>/>		>/>	>/>/>	>/>/>	>/>/>
	-1/-1/-1	11/6/5	1/2/2	1/4/3	NC/4/11	19/22/20	1/1/NC	21/12/16	17/17/21
	-2/-2/-2	4/2/1		NC/NC/2	1/3/2	1		2	3
DAY 3	ND/ND/ND		>/>/>	>/>/>		>/>	>/>/>	>/>/>	>/>/>
	-1/-1/-1	11/11/7	2/14/2	1/5/3	4/8/11	19/22/20	1/1/NC	27/16/20	30/30/26
	-2/-2/-2	4/2/2		NC/NC/2	1/4/2	1		2	3

Table 29: Results of T2 at 15°C on PCA with N_2-flushing

Log forming units of the plates after three days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.7	3.5	3.2	3.6	4.0	2.7	4.0	4.2

Table 30: Log forming units at T2 with N_2-flushing

When looking at the results of T2 (9.25 hours after To), there are still not lots of differences or changes. The log forming units decreased a little bit for CONTROL, HT,H and T. It's strange that the initial microflora in the CONTROL-bottle decreased. This may be little faults in diluting or inaccuracy during the experiment. The lactoperoxidase-system is activated in the test-bottles with HT, T and H, but the influence of the lactoperoxidase-system is not big at all. It has a little bactericidal effect on the total aerobic colony count.

In the CONTROL-bottle with N_2-flushing and in the bottle HN, the log forming units stay constant, there is no difference between T1 and T2, so there is a bacteriostatic effect. The bottle with thiocyanate and N_2 (TN), shows a little increase, so this condition isn't efficient at all. The most important result, is the decrease of the log forming units in the bottle with thiocyanate, H_2O_2 and N_2. There is a decrease from 3.9 log forming units (T1) to 2.7 log forming units (T2). As written in the tests above, adding both substrates is most efficient to activate the lactoperoxidase-system. Adding both substrates and N_2-flushing is even more efficient here.

- Results of the plates made 24 hours after To (9u15-24/3/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	-1/-1/-1	>/>/>	4/3/3	>/>/>	>/>/>	82/51/47	1/5/1	17/24/13	48/37/50
	-2/-2/-2	>/>/>	1/NC/NC	23/12/15	>/>/>	2/5/3	NC/NC/1	NC/1/3	7/3/NC
DAY 2	-1/-1/-1	>/>/>	7/4/5	>/>/>	>/>/>	>/>/>	4/5/2	17/24/21	48/37/50
	-2/-2/-2	>/>/>	2/NC/NC	24/18/18	>/>/>	5/7/6	NC/NC/2	NC/4/6	8/5/6
DAY 3	-1/-1/-1	>/>/>	9/4/6	>/>/>	>/>/>	>/>/>	4/7/2	23/35/30	48/37/50
	-2/-2/-2	>/>/>	2/1/NC	23/12/15	>/>/>	5/7/7	NC/NC/2	NC/4/8	10/6/8
	-3/-3	30/34			57/41				
	-4/-4	0/3			6/6				
	-5/-5	NC/NC			3/5				

Table 31: Results 24 hours after To at 15°C on PCA with N_2-flushing

Log forming units of the plates after three days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	6.2	3.5	4.9	6.4	4.5	3.3	4.2	4.6

Table 32: Log forming units 24 hours after To with N_2-flushing

The results of the plates made 24 hours after To, show more differences and changes. There is already growth in a few conditions. There is an increase of the total aerobic colony count in the CONTROL-bottle. In the conditions with H, T, CN, HTN, HN and TN, there is also growth of the total aerobic colony count, so the activation of the lactoperoxidase-system is gone and N_2-flushing has no more effect. In the bottle with both substrates (HT), the

lactoperoxidase-system is still activated after 24 hours at 15°C and has a bacteriostatic effect on the total aerobic colony count.

In the test with N_2-flushing, there are also differences. There is growth of the microflora in the CONTROL with N_2-flushing. So adding N_2 to raw milk, hasn't any bacteriostatic or bactericidal effect on the microflora. There is little growth of the microflora in the bottles HTN, HN and TN, although the log forming units, count in HTN are still lower than the initial microflora. So the system with both substrates (thiocyanate and H_2O_2) and N_2-flushing stays the most effective for pulling down the microbial growth.

So after 24 hours, only in the bottle with both thiocyanate and H_2O_2, the lactoperoxidase-system is still activated, but the bottle with HTN still shows the lowest quantities, even there is little growth here.

- Results of the plates made 48 hours after To (9u15-25/3/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	-1/-1		32/37				>/>		
	-2/-2		7/9			31/49	5/8	30/47	>/>
	-3/-3		3/1	>/>		5/3	NC/NC	4/1	8/14
	-4/-4			>/>		NC/NC		1/1	2/NC
	-5/-5	43/43		37/25	30/27				
	-6/-6	10/7			3/2				
	-7/-7	NC/NC			NC/NC				
DAY 2	-1/-1		47/45				>/>		
	-2/-2		7/11			31/49	5/8	34/69	>/>
	-3/-3		3/1	>/>		10/14	NC/NC	4/1	12/19
	-4/-4			>/>		NC/NC		1/1	2/NC
	-5/-5	43/43		37/25	30/27				
	-6/-6	10/7			3/2				
	-7/-7	NC/NC			1/NC				

Table 33: Results of the plates 48 hours after To at 15°C on PCA with N_2-flushing

Log forming units of the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	8.6	4.7	8.2	8.2	5.8	4.5	5.4	5.9

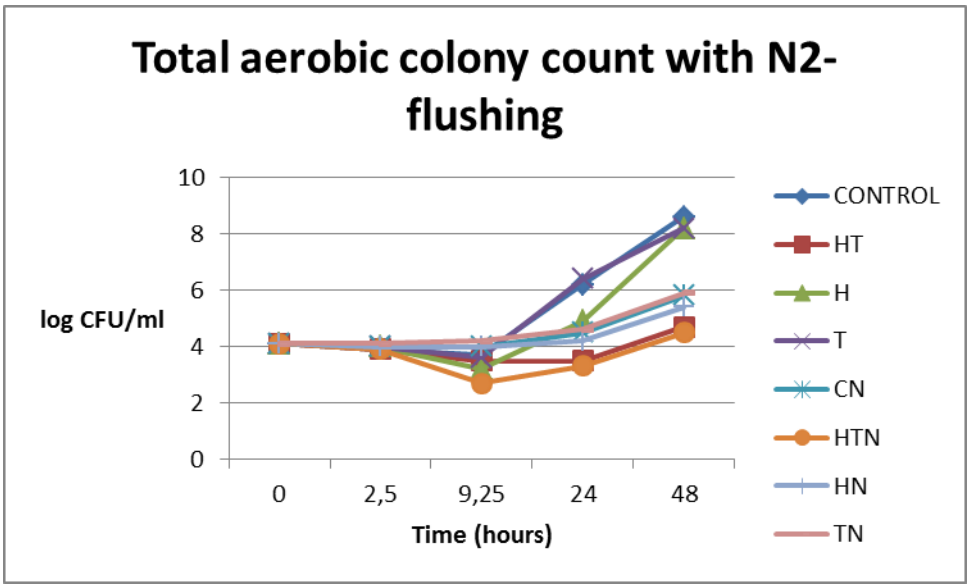
Table 34: Log forming units 48 hours after To with N_2-flushing

The results of the total aerobic colony counts 48 hours after To, are written above in table 33 and 34. The results of the plates made 48 hours after To, show growth in each condition. The results show that the growth increased a lot in the bottles CONTROL, H and T. So there is no more activation of the lactoperoxidase-system in the bottles with only thiocyanate or only H_2O_2 added. The log forming units are almost the same for the three conditions. For the bottle with both substrates added, there is also growth of the total aerobic colony count, so the lactoperoxidase-system is no more active after 48 hours. But important to mention is that the log forming units counted in this condition, are still a lot lower in comparison with the other conditions.

In the conditions with N_2-flushing, there is also growth of the total aerobic colony count, but the growth is lower than without N_2-flushing. So this is also positive. The growth in the bottle with both substrates and N_2-flushing, stays the lowest. After 48 hours, this system is still efficient. But there is no more activation of the lactoperoxidase-system in this condition, so there is a little growth.

So after 48 hours, the lactoperoxidase-system is no more active and N_2-flushing lost his effect on the total aerobic colony count. Important to mention is that condition HTN and HT stay the most efficient.

- Graphic



Graphic 9: Total aerobic colony count with N_2-flushing

The graphic shows results with almost no differences, that's what's written above. Up to 9.25 hours after To, results in all conditions are almost the same, they fall almost together. The total aerobic colony count here is quite low. After 24 hours and 48 hours, there are more differences. The bottles with HT and HTN show for all counting points, the lowest quantities of Colony Forming Units. They keep the bacterial count low, but they can't avoid growth after 48 hours. After 48 hours, the lactoperoxidase-system has no more activity at 15°C. Following conditions show the largest growth in each counting point: CONTROL, H and T. The conditions with N_2-flushing show lower Colony Forming Units.

4.4.2 Colony forming units on MRS

- Results of the plates made at To (9h15 – 23/3/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	18/17/18							
	-1/-1/-1	2/4/2							

Table 35: Results of To at 15°C on MRS with N_2-flushing

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	2.9							

Table 36: Log forming units at To with N_2-flushing on MRS

The results in table 35 and 36, give the initial microflora of *Lactobacillus* spp. in the raw milk at To. The quantity count here, is low. This is the reference. This is already a good beginning. There is no counting point after 2h30.

- Results of the plates made at T2 (18h30-23/3/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	16/18/15	>/>/>	25/18/14	37/34/31	>/>/20	16/14/12	20/16/24	31/26/26
	-1/-1/-1	3/4/4	4/1/3	4/4/5	3/8/NC	4/3/2	5/NC/NC	9/3/5	4/4/4

Table 37: Results of T2 at 15°C with N_2-flushing on MRS

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	2.9	3.1	3.0	3.2	3.2	2.8	3.0	3.1

Table 38: Log forming units at T2 with N_2-flushing on MRS

The results shown above, are almost identical in each condition. After 9,25 hours, there is almost no growth of the initial microflora of *Lactobacillus* spp. present at To.

The CONTROL-bottle shows a stable value. The colony forming units here stay the same as at To. In this case, the natural lactoperoxidase-system could be activated by the natural presence of thiocyanate and H_2O_2. The condition with both substrates added and N_2-flushing, is the only condition with decrease of the counting units. This explains the bactericidal effect on *Lactobacillus* spp. Again, this condition shows the best effect and result, which was the same on PCA. All other conditions (bottles with HT,H, T, CN, HN and TN) show increase of the growth of *Lactobacillus* spp. After 9.25 hours, this conditions have no more effect on *Lactobacillus* spp., so they can't be used.

- Results of the plates made 24 hours after To (9h15-24/3/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	>/>/>	23/25/22	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>
	-1/-1/-1	38/39/27	5/11/5	12/14/16	46/22/27	10/18/22	4/4/4	13/10/11	14/21/15

Table 39: Results 24 hours after To at 15°C with N_2-flushing on MRS

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	4.2	3.1	3.8	4.2	3.9	3.3	3.8	3.9

Table 40: Log forming units 24 hours after To with N_2-flushing on MRS

The quantities of *Lactobacillus* spp. 24 hours after To, show little changes. Almost in each condition, there is growth of the initial microflora of *Lactobacillus* spp. The growth is not high, but this shows that the lactoperoxidase-system doesn't work anymore after 24 hours. Also the N_2-flushing hasn't a bacteriostatic or bactericidal effect after 24 hours. Only the bottle HT, shows a bacteriostatic result. In this case, the lactoperoxidase-system is still active after

24 hours and there is no growth. The other conditions show no more efficiency after 24 hours, but this could already be concluded after 9.25 hours.

- Results of the plates made 48 hours after To (9h15-25/3/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND								
	-1/-1	>/>	55/65	>/>	>/>	>/>	66/65	>/>	>/>
	-2/-2	>/>	11/7	45/49	>/>	39/43	11/8	14/20	38/41
	-3/-3	33/36	NC/NC	6/10	26/27	6/8	2/2	3/3	10/7

Table 41: Results 48 hours after To at 15°C with N_2-flushing on MRS

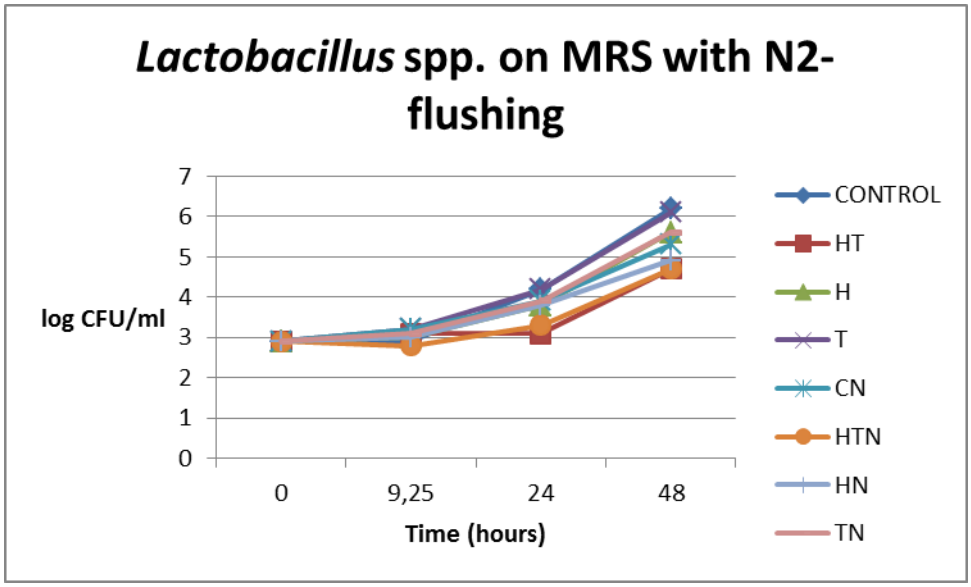
Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	6.2	4.7	5.6	6.1	5.3	4.7	4.9	5.6

Table 42: Log forming units 48 hours after To with N_2-flushing on MRS

The results of the plates made 48 hours after To, show increasing growths in each condition. After 48 hours, the lactoperoxidase-system has no more activation and the N_2-flushing has no more effect. Just one important thing to say, is that the growth of *Lactobacillus* spp. is the lowest in the bottles HT and HTN. This is similar to all results already described in this test. So these conditions are most efficient. So this conditions stay the most efficient, even after 48 hours.

- Graphic



Graphic 10: *Lactobacillus* spp. on MRS with N_2-flushing

The results of the Colony Forming Units of *Lactobacillus* spp. don't show lots of differences. At 9.25 hours after To, the results fall almost together for all conditions. After 24 hours, there are little differences, but HT and HTN show the lowest CFU's. At this moment, there is a little growth of the initial microflora. After 48 hours, there is more growth, but HT and HTN are still showing the lowest growths.

4.4.3 Colony forming units on M17

- Results of the plates made at To (9h15 – 23/3/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	27/29/27							
	-1/-1/-1	9/5/6							

Table 43: Results of To at 15°C with N_2-flushing on M17

Log forming units on the plates after 3 days: (calculation same as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.1							

Table 44: Log forming units at To with N_2-flushing on M17

When looking to the log forming units of *Streptococcus* spp. at To on M17, the quantity is rather low. There is an initial microflora of 3.1 log forming units. This is a good average result, what means there are not lots of *Streptococcus* spp. present. There is no counting point after 2h30.

- Results of the plates made at T2 (18h30-23/3/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	29/31/24	24/38/14	32/21/>	46/44/34	49/47/38	32/22/25	45/43/43	36/43/41
	-1/-1/-1	6/6/2	1/7/6	5/2/3	4/8/9	12/5/4	9/6/12	3/1/5	5/6/4

Table 45: Results of T2 at 15°C with N_2-flushing on M17

Log forming units on the plates after 3 days: (calculation same as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.1	3.1	3.1	3.5	3.5	3.1	3.3	3.3

Table 46: Log forming units at T2 with N_2-flushing on M17

The results of the plates made 9.25 hours after To are shown in table 45 and table 46. There are almost no changes in quantities count. Only the conditions T, CN, HN and TN show little growth of *Streptococcus* spp. In the bottle with only T, the lactoperoxidase-system isn't activated. Growth is possible. So adding just thiocyanate to the raw milk, isn't efficient and doesn't activate the lactoperoxidase-system. In the bottles CN, HN and TN, substrates didn't activate the lactoperoxidase-system and the N_2-flushing even hasn't any influence on the bacterial growth. So they aren't efficient to control the growth of *Streptococcus* spp.

The Colony Forming Units count in the bottles CONTROL, HT, H and HTN are the same as the initial microflora of *Streptococcus* spp. at To. In the bottles HT and H, the lactoperoxidase-system is activated and has a bacteriostatic effect on *Streptococcus* spp. after 9.25 hours. Also condition HTN shows the bacteriostatic effect of the activated lactoperoxidase-system and the influence of N_2-flushing on *Streptococcus* spp.

Controversial here, are the log forming units count in CONTROL after 9.25 hours. This is identical to the initial microflora, despite the absence of any substrates to activate the lactoperoxidase-system. In this case, the natural lactoperoxidase-system may be activated by the natural presence of thiocyanate and H_2O_2 in the raw milk.

- Results of the plates made 24 hours after To (9h15-24/3/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	>/>/>	39/37/32	>/>/>	>/>/>	>/>/>	31/33/30	>/>/>	>/>/>
	-1/-1/-1	37/34/43	4/3/NC	14/16/24	34/36/43	14/16/9	9/7/4	7/11/4	16/10/17

Table 47: Results 24 hours after To at 15°C with N_2-flushing on M17

Log forming units on the plates after 3 days: (calculation same as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	4.3	3.3	4.0	4.3	3.8	3.2	3.6	3.9

Table 48: Log forming units 24 hours after To with N_2-flushing on M17

The results of the plates made, 24 hours after To, are shown in table 47 and table 48. There is an increase in growth of *Streptococcus* spp. in each condition. So roughly said, the lactoperoxidase-system and N_2-flushing have no more effect after 24 hours. One remark, there is just a very small growth of *Streptococcus* spp. in conditions HT and HTN. So there is a delayed growth in these conditions after 24 hours. So these conditions stay the most efficient.

- Results of the plates made 48 hours after To (9h15-25/3/2015) AFTER 3 DAYS

	Dilution	CONTROL	CN	HTN
DAY 3	ND/ND			
	-1/-1	>/>	>/>	>/>
	-2/-2	>/>	45/57	9/9
	-3/-3	62/63	7/7	NC/2

Table 49: Results 48 hours after To at 15°C with N_2-flushing on M17

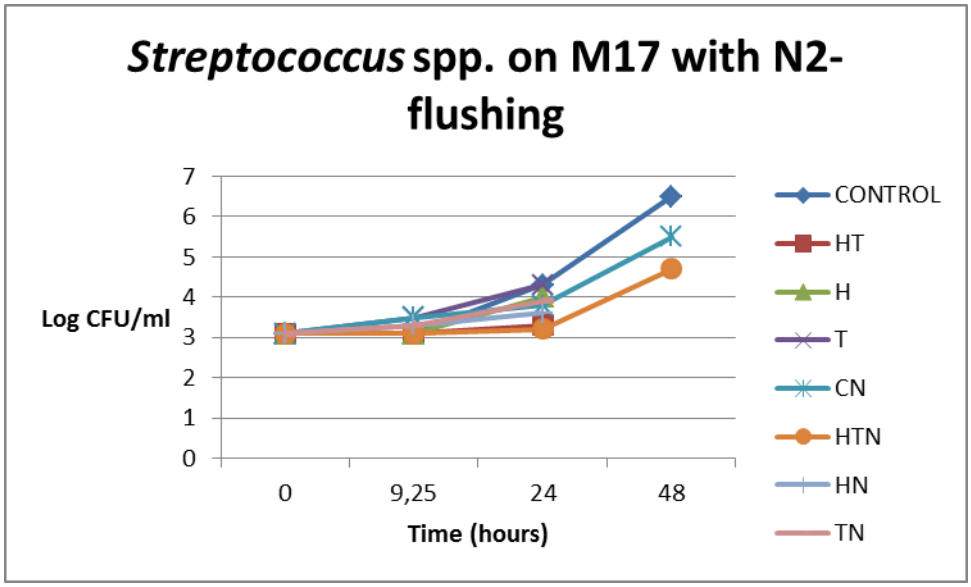
Log forming units on the plates after 3 days: (calculations same as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	6.5				5.5	4.7		

Table 50: Log forming units 48 hours after To with N_2-flushing on M17

There are only plates made from CONTROL, CN and HTN, 48 hours after To. There is an increase of the growth of *Streptococcus* spp. in each condition. So the N_2-flushing and the lactoperoxidase-system have no more influence on the bacterial growth 48 hours after To.

- Graphic



Graphic 11: *Streptococcus* spp. on M17 with N_2-flushing

As described above, the results for each condition don't show big differences up to 9.25 hours after To. The results of the plates 24 hours and 48 hours made after To, show an increase of the growth of *Streptococcus* spp. and in this way, they show the lack of working of the lactoperoxidase-system and N_2-flushing after 24-48 hours. HTN stays the best condition to minimalize the growth of *Streptococcus* spp.

4.5 Results of the second lactoperoxidase-test at 15°C with N_2-flushing

In this second lactoperoxidase-experiment at 15°C with N_2-flushing, the same procedure is used to confirm the results of the first experiment. Eight conditions are put into a warm water bath at 15°C. At different times, plates are made of PCA, MRS and M17. Here, the results are discussed.

4.5.1 Colony forming units on PCA (Plate Count Agar)

- Results of the plates made at To (9h40- 8/4/2015) AFTER 3 DAYS

DAY 3	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	ND/ND/ND	3/3/6							
	-1/-1/-1	5/6/1							

Table 51: Results of To at 15°C with N_2-flushing

Log forming units of the plates after 3 days

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.3							

Table 52: Log forming units at To on PCA with N_2-flushing

The initial microflora of the total aerobic colony count is 3.3 log forming units. This quantity at To is low. The reason for this low quantity of the total aerobic colony count at To is: the raw milk came straight from the university farm. So the raw milk used for this experiment was very fresh. The raw milk used in the other experiments came from the lorries that pick up the raw milk after a few days on different farms. So contamination of the raw milk in this experiment is more difficult than in the case of the 'milk lorries'. In the milk lorries contamination happens more easily because there is raw milk from different farms with different qualities, contamination of the milk lorries is possible and the raw milk is less fresher. Sometimes raw milk is picked up two days after milking. In this case here, the raw milk came from one or more cows since a few hours, so the milk is very fresh.

- Results of the plates made at T1 (11h40-8/4/2015) AFTER 3 DAYS

DAY 3	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	ND/ND/ND	11/8/11	4/12/9	7/1/3	5/7/2	1/2/3	5/3/4	2/1/1	7/6/11
	-1/-1/-1	5/5/4	4/4/1	2/2/1	3/1/3	4/3/2	2/1/2	5/1/3	1/2/1

Table 53: Results of T1 at 15°C on PCA with N_2-flushing

Log forming units of the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.4	3.2	2.3	3.1	3.2	2.3	1.8	2.6

Table 54: Log forming units at T1 on PCA with N_2-flushing

Two hours after To, at T1, results of the log forming units don't differ a lot, but some conditions show more effective results. Table 54 shows the results of the plates made 2 hours after To. It seems the lactoperoxidase-system is activated by adding one or both substrates and the N_2-flushing also has an inhibitory effect on the total aerobic colony count. The CONTROL-bottle shows an increase of the total aerobic colony count, so there is growth after 2 hours. That's normal because there are no substrates added, so no lactoperoxidase-system can be activated and there is no N_2-flushing to inhibit the growth of the initial microflora. The growth is very small, but there are just two hours between To and T1. All other conditions, show a bactericidal effect 2 hours after To. In this experiment, conditions H, HTN, HN and TN seem to have the best bactericidal effect on the total aerobic colony count. The first experiment shows similarly results, there is also a decrease in almost all conditions, but the decrease is very low because of the few hours between To and T1.

The quantities counted and showed in table 53 and 54 stay very low, because of the good quality of the raw milk.

- Results of the plates made at T2 (18h30-8/4/2015) AFTER 3 DAYS

DAY 3	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	ND/ND/ND	5/NC/1	NC/NC/1	1/1/3	2/1/NC	2/2/1	3/3/7	1/2/2	2/1/NC
	-1/-1/-1	NC/NC/NC	1/1/3	NC/NC/1	NC/NC/2	NC/3/3	2/2/3	2/1/3	1/4/4

Table 55: Results of T2 at 15°C on PCA with N_2-flushing

Log forming units of the plates after three days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	2.0	2.9	1.9	1.7	1.9	2.3	1.9	3.2

Table 56: Log forming units at T2 with N_2-flushing

Table 56 shows the log forming units of the total aerobic colony counts of the plates made 8.8 hours after To. At this point, there are different results for each condition. In first case, condition HTN shows a stable result, there is no difference between T1 and T2, so there is a

bacteriostatic effect. This is not bad, because no growth is possible after 8.8 hours when both substrates and N_2-flushing are added. Conditions CONTROL, HT, H, T and CN show decrease of the total aerobic colony count, so there is a bactericidal effect. It's strange that there is a decrease in the log forming units of condition CONTROL, because no lactoperoxidase-system can be activated. The reason here can be inaccuracy, or wrong dilutions. When adding one or both substrates seems to be positive to still activate the lactoperoxidase-system after 8.8 hours. Just adding N_2-flushing also shows a good result of inhibitory effect on the total aerobic colony count. Conditions HN and TN, have no more bacteriostatic or bactericidal effect on the total aerobic colony count. So this is important to keep in mind. In the first experiment, there was a decrease in the total aerobic colony count in each condition, with exception of condition TN. So as described here, condition TN, seems to be not effective. Even as HN, which shows a bacteriostatic effect in the first experiment at T2. So TN and HN, are less effective than the other conditions.

- Results of the plates made 24 hours after To (9h40-9/4/2015) AFTER 3 DAYS

DAY 3	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	ND/ND/ND	9/14/15	7/10/13	13/16/14	15/17/22	1/2/6	1/2/2	NC/1/3	1/1/2
	-1/-1/-1	2/7/8	7/10/11	13/13/15	16/17/19	NC/2/4	1/2/3	2/3/3	NC/3/4

Table 57: Results 24 hours after To at 15°C on PCA with N_2-flushing

Log forming units of the plates after three days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.5	3.7	3.8	3.9	3.0	3.0	3.1	3.1

Table 58: Log forming units 24 hours after To with N_2-flushing

In table 57 and 58 results are shown of the PCA-plates made 24 hours after To. The results show increase of the total aerobic colony count in each condition, with exception of condition TN. Here, there is a controversial with the words described above, because there is a small decrease of the total aerobic colony count in this situation. To make the right conclusion, comparisons have to be made with other similar experiments. The other conditions show small growth, so no more lactoperoxidase-system is activated or N_2-flushing has no more inhibitory effect after 24 hours. That's also what's written in the literature study, keeping raw milk at 15°C, activates the lactoperoxidase-system for 24-26 hours when one or both substrates are added. Here, after 24 hours, the lactoperoxidase-system has no more activation. Even N_2-flushing doesn't inhibit the growth after 24 hours. But the conditions with N_2-flushing and one or both substrates added, show the lowest quantities, even lower than the initial microflora, so these conditions are most effective after 24 hours. In the first experiment, there was also growth of almost all conditions, identical as described in this experiment. And also the conditions with N_2-flushing and adding one or both substrates, showed the lowest quantities.

- Results of the plates made 48 hours after To (9h40-10/4/2015) AFTER 3 DAYS

DAY 3	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	ND/ND/ND					>/>/>	>/>/>	>/>/>	>/>/>
	-1/-1/-1	>/>/>	>/>/>	>/>/>	>/>/>	34/36/43	50/52/55	>/>/>	>/>/>
	-2/-2/-2	>/>/>	>/>/>	>/>/>	>/>/>				

Table 59: Results of the plates 48 hours after To at 15°C on PCA with N_2-flushing

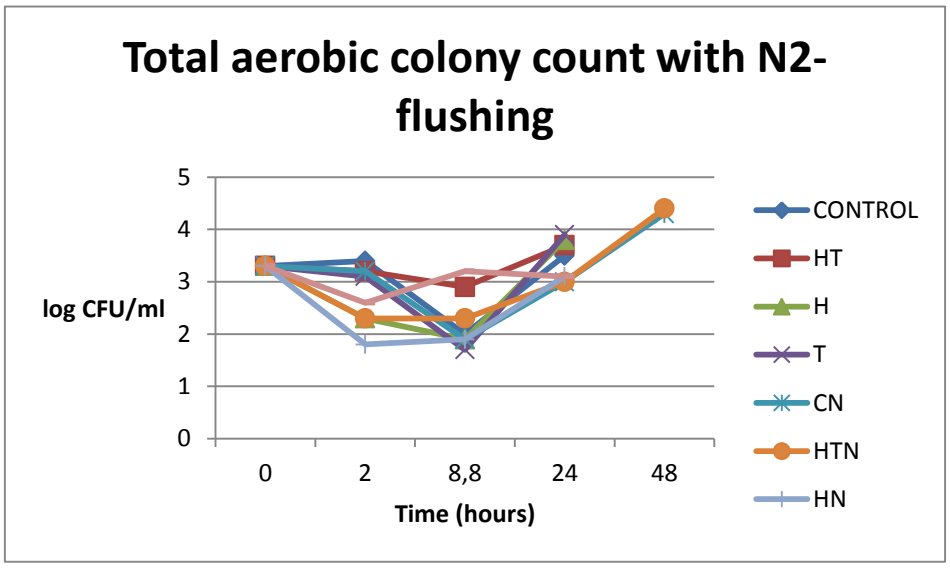
Log forming units of the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	>	>	>	>	4.3	4.4	>	>

Table 60: Log forming units 48 hours after To with N_2-flushing

Table 60 shows the log forming units of the plates made 48 hours after To. Only in conditions CN and HTN, countable quantities were possible. In all other conditions, the growth was too high to count any colonies. So holding raw milk 48 hours at 15°C, is no possibility to hold the total aerobic colony count low. In the first experiment, there was also growth 48 hours after To, but in that experiment, HTN and HT showed the best results, still after 48 hours.

- Graphic



Graphic 12: Total aerobic colony count with N_2-flushing at 15°C

Graphic 12 gives an overview of the results of the eight conditions on PCA at different counting points. The total aerobic colony count is low at To, but after 2 hours and after 8.8 hours at 15°C, there is a decrease of the total aerobic colony count, what shows even lower results of log forming units. After 24 hours there is already growth of the total aerobic colony count and after 48 hours, the results are so high, what means holding raw milk longer than 24 hours at 15°C only has negative results on the total aerobic colony count of the raw milk.

4.5.2 Colony forming units on MRS

- Results of the plates made at To (9h40 – 8/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	NC/NC/2							
	-1/-1/-1	NC/NC/NC							

Table 61: Results of To at 15°C on MRS with N_2-flushing

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	1.5							

Table 62: Log forming units at To with N_2-flushing on MRS

Table 62 shows the initial microflora of *Lactobacillus* spp. at To. The initial microflora of *Lactobacillus* spp. on MRS is 1.5 log forming units. This is a low quantity in comparison with the first experiment. The freshness of the raw milk is the reason for this low value at To. That's also described above.

- Results of the plates made at T1 (11h40-8/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>
	-1/-1/-1	5/14/18	6/7/8	9/18/21	4/4/9	13/14/16	7/11/12	7/8/11	15/15/16

Table 63: : Results of T1 at 15°C with N_2-flushing on MRS

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.8	3.5	3.9	3.5	3.9	3.7	3.6	3.9

Table 64: Log forming units at T1 with N_2-flushing on MRS

Tables 63 and 64, show the results of the plates made 2 hours after To. At T1, there is growth of *Lactobacillus* spp. on MRS in each condition. There is lot of growth, what's strange because there are just 2 hours between To and T1. So the lactoperoxidase-system has no bacteriostatic or bactericidal effect and N_2-flushing has no inhibitory effect on *Lactobacillus* spp. after holding raw milk 2 hours at 15°C.

Even there is growth, the counted quantities stay low.

- Results of the plates made at T2 (18h30-8/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	>/>/>	NC/NC/NC	>/>/>	>/>/>	>/>/>	8/9/10	>/>/>	>/>/>
	-1/-1/-1	1/4/9	NC/NC/NC	7/10/11	4/4/5	13/14/15	1/2/3	9/9/10	12/17/18

Table 65: Results of T2 at 15°C with N_2-flushing on MRS

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.4	0	3.7	3.3	3.8	3.0	3.7	3.9

Table 66: Log forming units at T2 with N_2-flushing on MRS

When looking to the results of the plates made 8.8 hours after To, interesting results are shown in tables 65 and 66. Condition HN still shows growth of *Lactobacillus* spp. Condition TN shows a stable quantity, which means there is no growth between T1 and T2. So in this condition N_2-flushing has a bacteriostatic effect on *Lactobacillus* spp. In all other conditions, there is a decrease of the log forming units. This is strange, because after 2 hours at T1, there was growth. So the lactoperoxidase-system needed time to be activated and N_2-flushing even had immediately an inhibitory effect. When looking to the results of T2 of the first experiment, there was a small growth of *Lactobacillus* spp. at this moment, which agrees with these results of the second experiment.

In table 66, conditions HT and HTN show the most effective results after 8.8 hours with the lowest log forming units count.

- Results of the plates made 48 hours after To (9h40-10/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND					>/>/>	>/>/>	>/>/>	>/>/>
	-1/-1/-1	>/>/>	>/>/>	>/>/>	>/>/>	7/10/12	10/20/16	14/16/16	15/16/23
	-2/-2/-2	75/78/86	56/66/72	95/96/121	94/98/112				

Table 67: Results 48 hours after To at 15°C with N_2-flushing on MRS

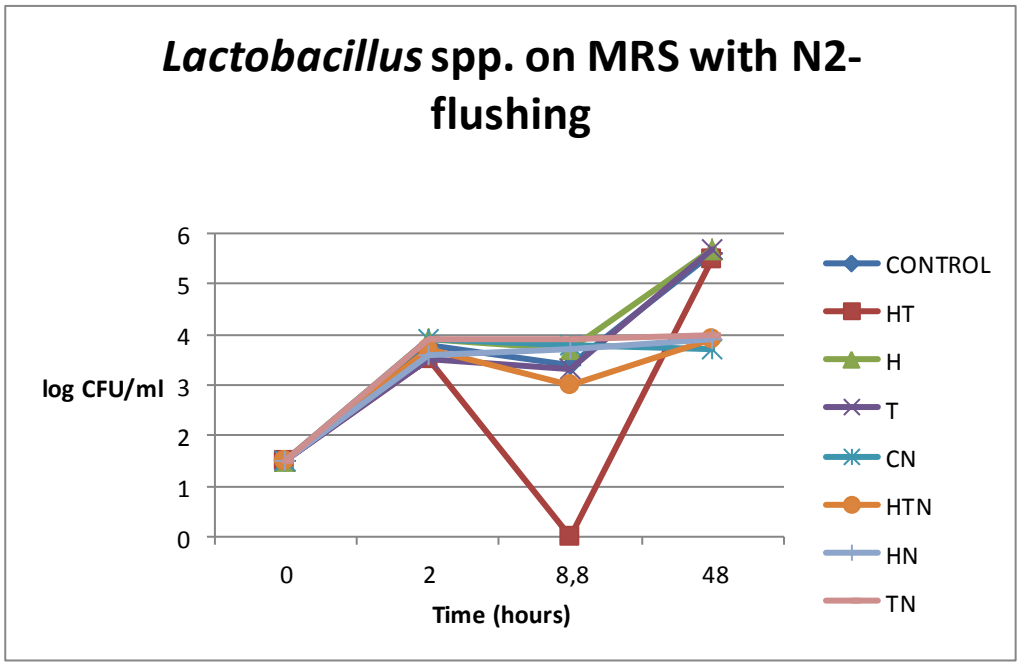
Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	5.6	5.5	5.7	5.7	3.7	3.9	3.9	4.0

Table 68: Log forming units 48 hours after To with N_2-flushing on MRS

Table 68 shows the growth of *Lactobacillus* spp. in each condition, when the raw milk is kept 48 hours at 15°C. After holding the raw milk 48 hours at 15°C, there is growth of *Lactobacillus* spp. in each condition. So there is no more activation of the lactoperoxidase-system, that's also what's written in the literature study. After 24-26 hours, there is no more activation of the lactoperoxidase-system when holding raw milk at 15°C. N_2-flushing even has an inhibitory effect after 48 hours. But the conditions with N_2-flushing and one or both substrates added, show lowest growths. So these conditions are most effective after 48 hours, even there is growth. In the first experiment, there is also growth in each condition, but conditions HT and HTN were most effective. So a third experiment has to confirm these conclusions.

- Graphic



Graphic 13: *Lactobacillus* spp. on MRS with N_2-flushing at 15°C

Graphic 13 shows a comparison between the eight conditions for the growth of *Lactobacillus* spp. at 15°C. Quantities are low because of the low initial microflora. After 8.8 hours there is a decrease of the Colony Forming Units. But after 48 hours, there is an increase of the Colony Forming Units, which means there is growth and the lactoperoxidase-system and N_2-flushing have no more bacteriostatic or bactericidal effect on *Lactobacillus* spp.

4.5.3 Colony forming units on M17

- Results of the plates made at To (9h40 – 8/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	33/37/39							
	-1/-1/-1	11/12/17							

Table 69: Results of To at 15°C with N_2-flushing on M17

Log forming units on the plates after 3 days: (calculation same as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.8							

Table 70: Log forming units at To with N_2-flushing on M17

When looking to the M17-plates in table 70, 3.8 log forming units is the initial microflora of *Streptococcus* spp. This is a moderate value, unless the raw milk is very fresh. There are more *Streptococcus* spp. present at To, than *Lactobacillus* spp. or the total aerobic colony count.

- Results of the plates made at T1 (11h40-8/4/2015) AFTER 3 DAYS

	Dilution	**CONTROL**	**HT**	**H**	**T**	**CN**	**HTN**	**HN**	**TN**
DAY 3	ND/ND/ND	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>
	-1/-1/-1	9/14/16	14/21/23	17/20/20	16/18/23	18/19/21	10/12/20	13/14/18	19/21/22

Table 71: Results of T1 at 15°C with N_2-flushing on M17

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	**HT**	**H**	**T**	**CN**	**HTN**	**HN**	**TN**
Log forming units after 3 days	3.8	4.0	4.0	4.0	4.0	3.8	3.9	4.0

Table 72: Log forming units at T1 with N_2-flushing on M17

After keeping the raw milk 2 hours at 15°C, following results are noted in tables 71 and 72. The CONTROL-bottle and the condition HTN, show stable results. HTN shows a bacteriostatic effect on *Streptococcus* spp. when both substrates and N_2-flushing are added. No growth is possible. Also the CONTROL-bottle shows the same result as the initial microflora. But there are just 2 hours between To and T1.

The other conditions show little growth of *Streptococcus* spp. The growth is not high, so there may be a little bacteriostatic effect of the lactoperoxidase-sytem and N_2-flushing.

- Results of the plates made 48 hours after To (9h40-10/4/2015) AFTER 3 DAYS

	Dilution	**CONTROL**	**HT**	**H**	**T**	**CN**	**HTN**	**HN**	**TN**
DAY 3	ND/ND/ND					>/>/>	>/>/>	>/>/>	>/>/>
	-1/-1/-1	>/>/>	>/>/>	>/>/>	>/>/>	55/56/58	74/76/92	64/76/80	68/78/87
	-2/-2/-2	76/88/120	85/87/70	78/80/96	110/116/133				

Table 73: Results 48 hours after To at 15°C with N_2-flushing on M17

Log forming units on the plates after 3 days: (same calculation as written above)

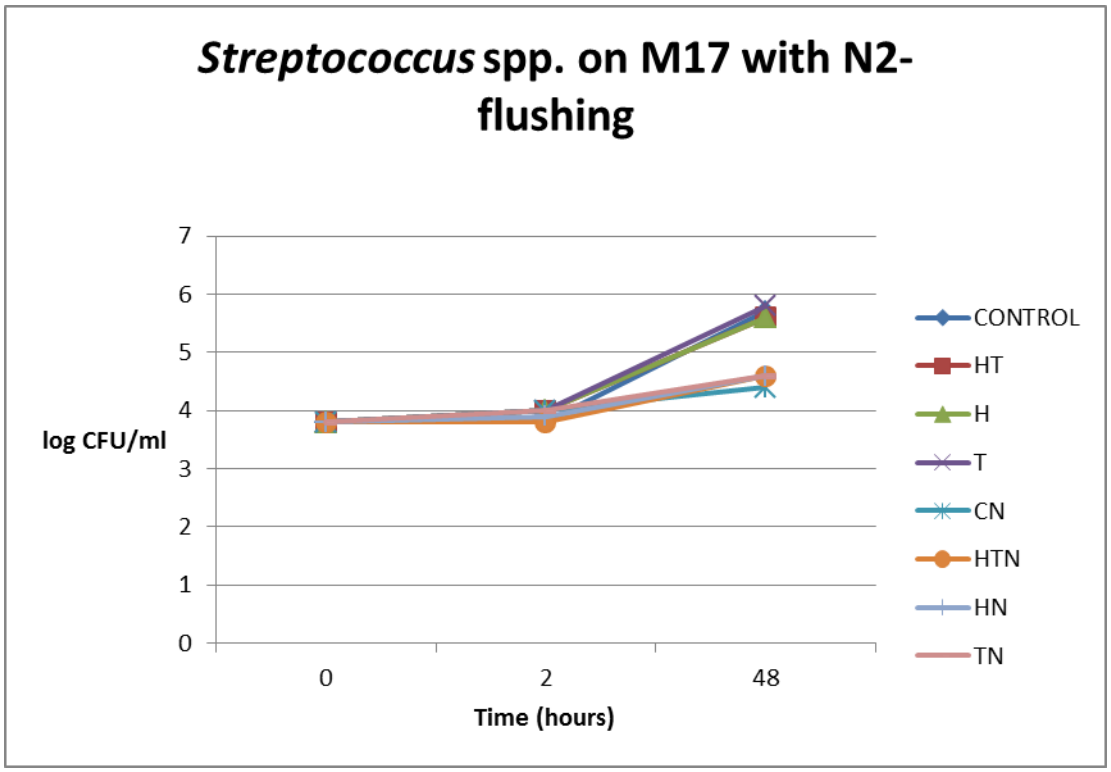
	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	5.7	5.6	5.6	5.8	4.4	4.6	4.6	4.6

Table 74: Log forming units 48 hours after To with N_2-flushing on M17

Holding the raw milk 48 hours at 15°C shows growth of *Streptococcus* spp. in all conditions. Results are noted in table 74. Despite there is growth, the log forming units stay the lowest in the conditions with N_2-flushing and one or both substrates added. So these conditions may be most efficient for controlling the growth of *Streptococcus* spp. This is also shown in the first experiment, where HTN is the best condition, even after 48 hours.

The conditions with N_2-flushing and adding one or both substrates are most effective to control the initial microflora of the total aerobic colony count and *Lactobacillus* spp. following this second experiment. After 24 hours at 15°C, there is growth on each growth medium, which is normal following the literature study. For *Streptococcus* spp., there is already growth after two hours, with exception of condition HTN. So to control the growth of *Streptococcus* spp., HTN is the best condition.

- Graphic



Graphic 14: *Streptococcus* spp. on M17 with N_2-flushing at 15°C

Graphic 14 doesn't show lots of changes between the colony forming units at To and T1 (after 2 hours). After 48 hours there is an increase of the log forming units of *Streptococcus* spp., so growth is possible when holding raw milk for 48 hours at 15°C. Unless there is growth after 48 hours, conditions CN, HTN, HN and TN still show moderate growths.

4.6 Results of the third lactoperoxidase-test at 15°C with N_2-flushing

A third and last experiment was set up at 15°C to confirm the statements made in the other two experiments at 15°C. In this way, results of the three experiments can be compared and a global conclusion can be made.

In this experiment, plates of PCA, MRS and M17 are made at different times. There are eight conditions, like in the other experiments: CONTROL, HT, H, T, CN, HTN, HN and TN.

4.6.1 Colony forming units on PCA (Plate Count Agar)

- Results of the plates made at To (9h00 – 20/4/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	ND/ND/ND	34/72/77							
	-1/-1/-1	7/23/26							
DAY 2	ND/ND/ND	>/>/43							
	-1/-1/-1	16/27/29							
DAY 3	ND/ND/ND	>/>/50							
	-1/-1/-1	16/31/29							

Table 75: Results of To at 15°C with N_2-flushing

Log forming units of the plates after 3 days

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	4.1							

Table 76: Log forming units at To on PCA with N_2-flushing

In table 75, results are noted of To on PCA. Each day, the Colony Forming Units were controlled and noted. At day one, there are already lots of colony counts, this doesn´t change a lot for the second and third day. After 3 days, the log forming units at To were calculated. There is a value of 4.1 log forming units for the initial microflora of the total aerobic colony count. This value is high so there is contamination of the raw milk or the raw milk isn´t very fresh at all. The milk is already a few days old or the milk comes from different farms. But the value stays acceptable.

The value of 4.1 log forming units at To is the same value as To in the first experiment. In this way, a good comparison can be made between the two experiments.

- Results of the plates made at T1 (11h30-20/4/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	ND/ND/ND	65/68/70	NC/NC/NC	52/55/60	63/68/69	52/60/70	>/>/>	61/62/65	62/64/76
	-1/-1/-1	10/32/24	1/NC/NC	3/5/6	10/11/13	13/18/24	1/6/10	16/20/24	17/22/23
DAY 2	ND/ND/ND	>/>/>	49/50/57	>/>/>	>/>/>	>/>/>	>/>/7	>/>/>	>/>/>
	-1/-1/-1	19/36/26	9/10/13	14/18/20	12/12/17	28/29/28	15/11/17	18/22/25	27/26/31
DAY 3	ND/ND/ND	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>	>/>/10	>/>/>	>/>/>
	-1/-1/-1	26/36/27	11/18/18	16/18/22	12/12/17	28/31/28	19/18/22	20/23/25	28/27/32

Table 77: Results of T1 at 15°C on PCA with N_2-flushing

Log forming units of the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	4.2	3.9	4.0	3.8	4.2	4.0	4.0	4.2

Table 78: Log forming units at T1 with N_2-flushing

In table 77, the total aerobic colony counts of the different conditions are noted of T1, made 2h30 after To. Each day changes in colony counts were noted, to become a final result after 3 days. Between day 1 and day 2, most changes can be seen in the total aerobic colony counts. There are not lots of differences between the results at day 2 and day 3.

In table 78, the log forming units are noted for each condition. At this moment, there is growth of the total aerobic colony count in the CONTROL-bottle, CN and TN. The growth is very small, there are just 2h30 hours between To and T1. In these three conditions, no bacteriostatic or bactericidal effect can be seen. The growth of the total aerobic colony count in the CONTROL-bottle is normal. There are no substrates added or there is no N_2-flushing, so there is no inhibition possible of the initial microflora. In the second experiment, there is also growth of the total aerobic colony count in the CONTROL-bottle after two hours, which is normal. The growth in CN and TN seems to be not very correct because there is N_2-flushing in both conditions and in condition TN, substrate thiocyanate is added. By following these results, only adding N_2-flushing is not enough to inhibit the growth of the total aerobic colony count. Condition TN isn't also usual to inhibit the initial growth. The other conditions show a very limited decrease of the log forming units between To and T1, so here the lactoperoxidase-system is activated by the substrates and N_2-flushing also plays an inhibitory role on the total aerobic colony count, 2h30 after To. So all other conditions have a bactericidal effect on the total aerobic colony count after holding raw milk 2h30 at 15°C.

- Results of the plates made at T2 (18h30-20/4/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	ND/ND/ND	>/>/>	NC/NC/NC	>/>/>	>/>/>	>/>/>	NC/NC/NC	>/>/>	>/>/>
	-1/-1/-1	25/34/42	NC/NC/NC	20/24/25	35/36/37	31/34/39	NC/NC/NC	24/28/30	27/33/36
DAY 2	ND/ND/ND	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>
	-1/-1/-1	31/42/48	28/25/25	22/31/34	40/40/43	34/36/42	21/26/33	33/36/37	30/37/40
DAY 3	ND/ND/ND	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>	>/>/>
	-1/-1/-1	31/42/50	34/33/27	23/32/35	41/40/43	36/36/43	25/29/36	37/39/37	32/38/40

Table 79: Results of T2 at 15°C on PCA with N_2-flushing

Log forming units of the plates after three days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	4.3	4.2	4.2	4.3	4.3	4.2	4.3	4.3

Table 80: Log forming units at T2 with N_2-flushing

In table 79, results are given of the plates made at T2, 9h30 after To. Each day, changes in the total aerobic colony counts of each plate were noted. The differences in counts were largest between day 1 and day 2. Between day 2 and day 3, there were almost no changes in the total aerobic colony counts of each condition.

In table 80, the log forming units of T2 are noted. There is growth in each condition. The increase of growth is not big, but there is growth, which means there is no more lactoperoxidase-activity after 9h30 at 15°C, and N_2-flushing hasn´t any inhibitory effect, following this experiment. That's strange because normally the lactoperoxidase-system should be activated, like in the other experiments. In the previous experiments, there was a decrease in the total aerobic colony counts at T2, which means the lactoperoxidase-system had a bactericidal effect on the total aerobic colony count and also N_2-flushing inhibits growth. With exception of condition TN in the first experiment. This condition also shows growth at T2. So when comparing the first and the third experiment, TN seems to be not effective, even after 9h30 hours.

- Results of the plates made 24 hours after To (9h00-21/4/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	ND/ND		>/>			>/>	>/>	>/>	>/>
	-1/-1		9/16	>/>		57/67	13/15	39/41	52/57
	-2/-2	>/>	4/NC	10/24	>/>	4/8	1/2	4/4	8/11
	-3/-3	45/49		1/1	52/62				
	-4/-4	7/10			3/9				
DAY 2	ND/ND		>/>			>/>	>/>	>/>	>/>
	-1/-1		12/17	>/>		57/67	28/15	>/>	>/>
	-2/-2	>/>	5/2	10/24	>/>	11/11	4/4	4/6	11/12
	-3/-3	>/>		1/2	52/62				
	-4/-4	7/11			3/9				
DAY 3	ND/ND		>/>			>/>	>/>	>/>	>/>
	-1/-1		15/19	>/>		>/>	30/16	>/>	>/>
	-2/-2	>/>	5/4	10/24	>/>	11/13	5/6	5/6	11/12
	-3/-3	>/>		1/2	>/>				
	-4/-4	7/11			3/9				

Table 81: Results 24 hours after To at 15°C on PCA with N_2-flushing

Log forming units of the plates after three days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	6.7	3.9	4.9	6.5	4.8	4.4	4.4	4.8

Table 82: Log forming units 24 hours after To with N_2-flushing

In table 81, the results are noted of the total aerobic colony counts of each condition, 24 hours after To. The colonies were count each day. Between day 1 and day 2, most differences can be seen, there is most growth. When looking to the results of day 2 and day 3, there are not lots of changes. The total aerobic colony counts stay almost identical.

In table 82, the log forming units of the total aerobic colony counts 24 hours after To are calculated. The total aerobic colony counts show growth in almost each condition. That's quite normal because of the scientific information in the literature study. There, it's written that the lactoperoxidase-system is activated for 24-26 hours when raw milk is kept at 15°C. Here, in this situation, after holding the raw milk 24 hours at 15°C, there is no more activation of the lactoperoxidase-system because of the growth of the total aerobic colony count in each condition. Unless there is growth, the conditions with N_2-flushing and adding one or both substrates, show the lowest growth, which means there is still inhibition of the growth by N_2-flushing. At T1 and T2, condition TN showed to be not effective, so conditions HTN and HN are really effective, at each counting point. That's also what's described in the previous experiments. In the first and third experiment, condition HT also showed good results. Here, condition HT still has a bactericidal effect after 24 hours. In the first experiment, it had a bacteriostatic effect after 24 hours. So in this condition, by adding both substrates there is still activation of the lactoperoxidase-system after 24 hours at 15°C. This condition still plays an important role in the experiment.

- Results of the plates made 48 hours after To (9h00-22/4/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	-1/-1		>/>	>/>		>/>	>/>	>/>	>/>
	-2/-2	>/>	49/59	>/>	>/>	>/>	11/18	47/46	>/>
	-3/-3/-3	>/>/>	8/5	>/>	>/>/>	21/16	NC/1	5/7	14/22
	-4/-4	>/>			>/>				
	-5/-5/-5	78/67/75			40/41/38				
DAY 2	-1/-1		>/>	>/>		>/>	>/>	>/>	>/>
	-2/-2	>	53/63	>/>	>	>/>	11/18	47/46	>/>
	-3/-3/-3	>/>/>	8/5	>/>	>/>/>	23/17	NC/1	5/7	14/22
	-4/-4/-4	>/>		82/73/87	>/>				
	-5/-5/-5	78/67/75		8/17/16	40/41/38				
DAY 3	-1/-1		>/>	>/>	>	>/>	>/>	>/>	>/>
	-2/-2	>	53/63	>/>	>/>/>	>/>	11/18	>/>	>/>
	-3/-3/-3	>/>/>	8/5	>/>	>/>	23/17	NC/1	5/7	14/22

	-4/-4/-4	>/>		82/73/87	40/41/38				
	-5/-5/-5	67/75/78		8/17/16					

Table 83: Results of the plates 48 hours after To at 15°C on PCA with N_2-flushing

Log forming units of the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	8.6	5.5	7.8	7.3	6.0	4.9	5.5	6.0

Table 84: Log forming units 48 hours after To with N_2-flushing

The results of the plates made 48 hours after To (20/4/2015) on PCA, are noted in table 83. Each day, changes in the total aerobic colony counts were detected, by controlling the plates and counting the colonies. There are not lots of changes in the colony counts during the three following days. The total aerobic colony counts stay almost the same, without any changes. The log forming units were calculated from the results at day 3 and noted down in table 84.

There is growth of the total aerobic colony count in each condition when the raw milk is kept 48 hours at 15°C. The lactoperoxidase-system doesn´t work anymore at this point and N_2-flushing has no more inhibitory effect after 48 hours. That's normal because of the scientific information about the lactoperoxidase-system in the literature study. Unless there is growth, conditions HT, HTN and HN show lowest quantities, which means these conditions are most effective to control the growth of the total aerobic colony count, even after 48 hours. When adding both substrates and N_2-flushing, seems to be the best condition to inhibit growth. That's also what's described in the first experiment.

- Results of the plates made 72 hours after To (9h00-23/4/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	-1/-1		>				>/>		
	-2/-2		>/>				>/>		
	-3/-3/-3		>/>			>	74/75	>/>/>	>
	-4/-4		63/80	>		>/>/>		>/>	>/>/>
	-5/-5/-5	>		62/51	>	30/40		15	29/34
	-6/-6/-6	35/40/27		10/7/4	18/22/14				

	-7/-7	5/3			2/4				
DAY 3	-1/-1		>				>/>		
	-2/-2		>/>				>/>		
	-3/-3/-3		>/>			>	74/75	>/>/>	>
	-4/-4/-4		63/80	>		>/>/>		>/>	>/>/>
	-5/-5/-5	>		62/51	>	30/40		15	29/34
	-6/-6/-6	35/40/27		10/7/4	18/22/14				
	-7/-7	5/3			2/4				

Table 85: Results of the plates 72 hours after To at 15°C on PCA with N_2-flushing

Log forming units of the plates after 3 days: (same calculation as written above)

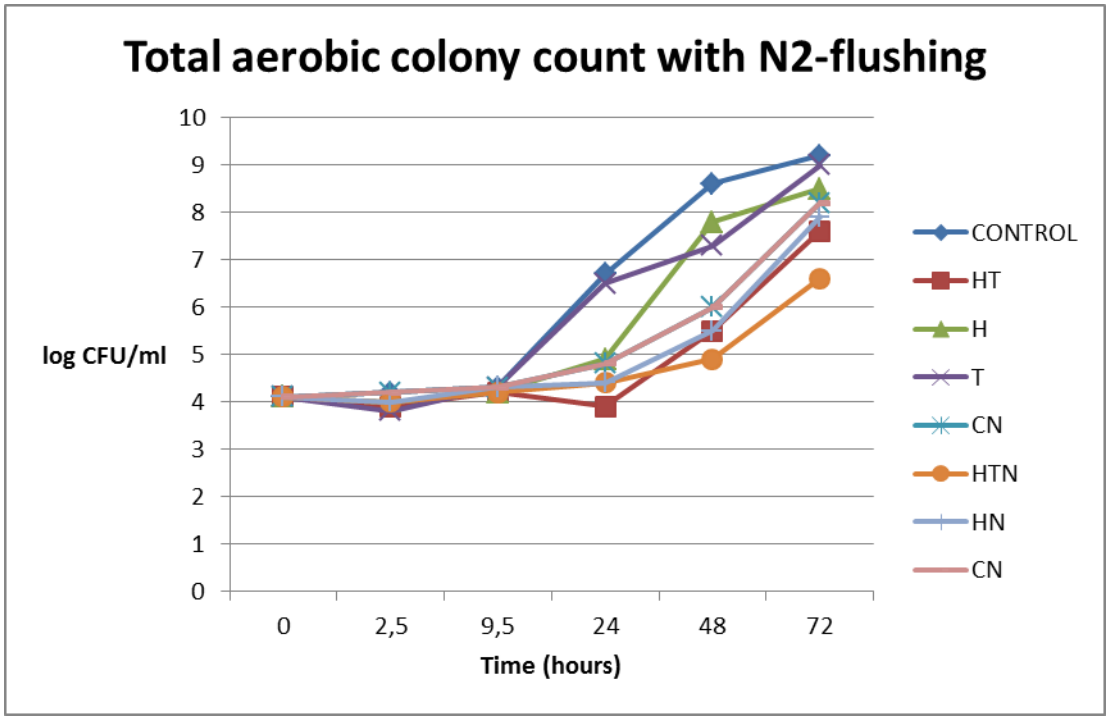
	CONTROL	**HT**	**H**	**T**	**CN**	**HTN**	**HN**	**TN**
Log forming units after 3 days	9.2	7.6	8.5	9.0	8.2	6.6	7.9	8.2

Table 86: Log forming units 72 hours after To with N_2-flushing

In table 85, results are noted of the plates made 72 hours after To (20/4/2015). There are only results of day 1 and day 3, because there was a weekend between the counting days. There are no changes in the total aerobic colony counts between day 1 and day 3.

In table 86, the results of the log forming units are noted, after calculating. There is growth in each condition when the raw milk is kept 72 hours at 15°C. When there is growth after holding raw milk 48 hours at 15°C, it's normal there is still growth of the total aerobic colony count 72 hours after To in each condition. Condition HTN stays most effective, even after 72 hours. The log forming units of the total aerobic colony count stay lowest in this situation.

- Graphic



Graphic 15: Total aerobic colony count with N_2-flushing at 15° C

In graphic 15, changes in total aerobic colony counts of each condition can be seen, at different times. Up to 9.5 hours after To, log forming units stay stable for each condition. After 24 hours, log forming units increase for each condition until 72 hours after To. In the graphic, the lines of condition HTN and HT show lowest quantities of the total aerobic colony counts, so these conditions are most effective, with HTN as the best condition to hold raw milk at 15°C.

4.6.2 Colony forming units on MRS

- Results of the plates made at To (9h00 – 20/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	>/>/>							
	-1/-1/-1	6/16/19							

Table 87: Results of To at 15°C on MRS with N_2-flushing

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.8							

Table 88: Log forming units at To with N_2-flushing on MRS

In table 87 and 88, the results are noted of the Colony Forming Units of *Lactobacillus* spp. at To after 3 days of incubation at 30°C. The initial microflora here is 3.8 log forming units. This value is high to start with. So the raw milk is contaminated or not fresh at all. This value stays moderate, but higher than the initial microflora in the first and second experiment, so here the raw milk is more contaminated.

- Results of the plates made 24 hours after To (9h00-21/4/2015) AFTER 3 DAYS

	Dilution	**CONTROL**	**HT**	**H**	**T**	**CN**	**HTN**	**HN**	**TN**
DAY 3	ND/ND	>/>	>/>	>/>	>/>	>/>	>/>	>/>	>/>
	-1/-1	>/>	14/16	25/29	>/>	25/32	9/16	20/20	34/37
	-2/-2	18/23	1/NC	2/1	33/35	4/6	4/NC	2/NC	1/3

Table 89: Results 24 hours after To at 15°C with N_2-flushing on MRS

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	**HT**	**H**	**T**	**CN**	**HTN**	**HN**	**TN**
Log forming units after 3 days	5.0	3.9	4.1	5.2	4.2	3.8	4.0	4.2

Table 90: Log forming units 24 hours after To with N_2-flushing on MRS

In table 89, the results of the colony counts of *Lactobacillus* spp. are noted of the point made 24 hours after To, counted after 3 days. The calculations of the log forming units are noted in table 90. There is growth of *Lactobacillus* spp. in almost all conditions, with exception of condition HTN. This condition shows a stable result, with no growth. So unless the lactoperoxidase-system is no more activated, N_2-flushing seems to inhibit the growth of *Lactobacillus* spp. in condition HTN. Condition HT shows very low growth, so unless there is growth, this condition is also an effective condition to keep the log forming units low of *Lactobacillus* spp. when holding raw milk 24 hours at 15°C. That's also what's described in the first experiment: conditions HT and HTN are very effective for *Lactobacillus* spp.

- Results of the plates made 48 hours after To (9h00-22/4/2015) AFTER 3 DAYS

	Dilution	**CONTROL**	**HT**	**H**	**T**	**CN**	**HTN**	**HN**	**TN**
DAY 3	-1/-1	>	>/>	>/>	>	>/>	57/61	>/>	>/>
	-2/-2	>/>	65/65	>/>	>/>	64/87	9/13	45/46	72/65
	-3/-3/-3	47/36/55	8/10	42/77	40/44/55	4/14	1/NC	1/4	7/12

Table 91: Results 48 hours after To at 15°C with N_2-flushing on MRS

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	6.4	5.7	6.5	6.4	5.6	4.7	5.4	5.5

Table 92: Log forming units 48 hours after To with N_2-flushing on MRS

In table 91, results are noted of the colony counts of *Lactobacillus* spp. after 3 days on MRS, when the raw milk is kept 48 hours at 15°C. Of these results, the log forming units are calculated and noted in table 92. There is growth of *Lactobacillus* spp. in each condition. So when the milk is kept 48 hours at 15°C, no more lactoperoxidase-system is activated or N_2-flushing has no more inhibitory effect. Conditions HTN and HN show lowest quantities of *Lactobacillus* spp., unless there is growth. So the same conclusion can be made as on PCA, HTN and HN are the best conditions after 48 hours at 15°C to hold the quantities of *Lactobacillus* spp. lower than the other conditions. That's also what's shown in the first and second experiment.

- Results of the plates made 72 hours after To (9h00-23/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	-2/-2		>/>			>/>	>/78	>/>	>/>
	-3/-3	>	>/>	>/>	>	>/>	16/15	77/86	>/>
	-4/-4	>/>	32/33	>/>	>/>	87/128	5/3	17/20	144/92
	-5/-5	67/75		46/49	30/43				
	-6	6			5				

Table 93: Results 72 hours after To at 15°C with N_2-flushing on MRS

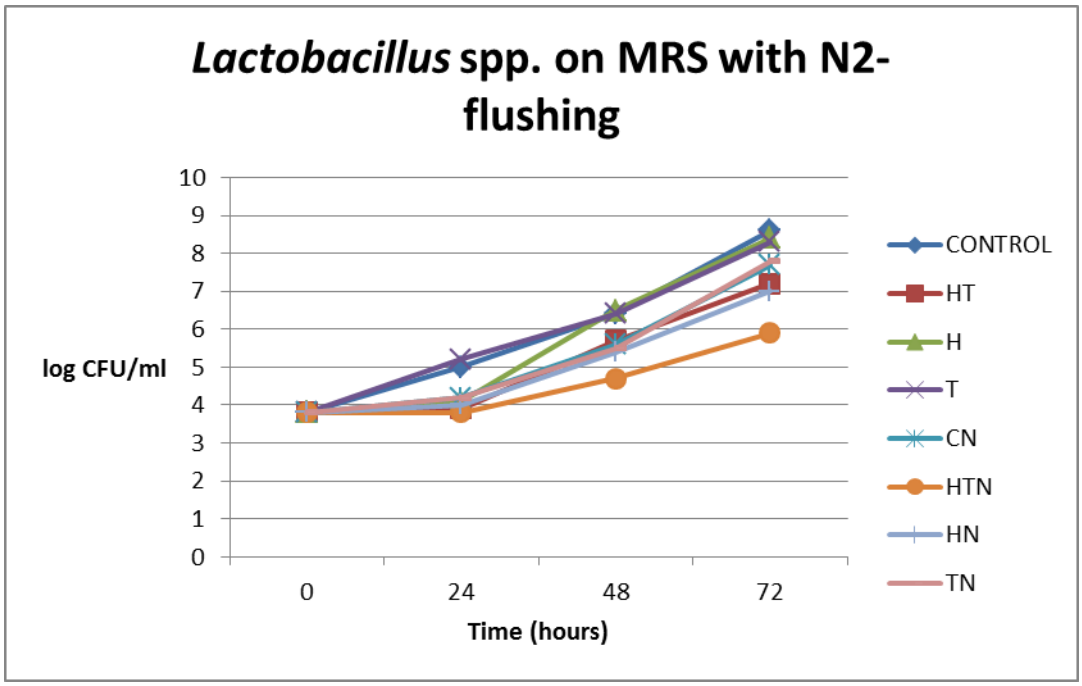
Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	8.6	7.2	8.4	8.3	7.7	5.9	7.0	7.8

Table 94: Log forming units 72 hours after To with N_2-flushing on MRS

In table 93, results are noted of the colony counts of *Lactobacillus* spp. on MRS, when the raw milk is kept 72 hours at 15°C. In table 94, the log forming units are noted. There is growth in each condition, but condition HTN shows the lowest growth of *Lactobacillus* spp. That's also what's described on PCA. So to control the growth of the total aerobic colony count and *Lactobacillus* spp., condition HTN is the best at each counting point.

- Graphic



Graphic 16: *Lactobacillus* spp. on MRS with N_2-flushing at 15° C

In graphic 16, results are given of the log forming units of *Lactobacillus* spp. in different conditions at different times, when the raw milk is kept at 15°C. HTN shows the best results, with a stable count between To and 24 hours after To. After 48 hours, there is also growth of *Lactobacillus* spp. in condition HTN, but the growth stays lowest of all conditions. In the other conditions, there is already growth after 24 hours at 15°C. And the log forming units keep increasing until 72 hours after To at 15°C.

4.6.3 Colony forming units on M17

- Results of the plates made at To (9h00 – 20/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	>/>/>							
	-1/-1/-1	9/10/13							

Table 95: Results of To at 15°C with N_2-flushing on M17

Log forming units on the plates after 3 days: (calculation same as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.7							

Table 96: Log forming units at To with N_2-flushing on M17

In table 95 and 96, the results are noted of the initial microflora of *Streptococcus* spp. in the raw milk at To. This value is high, even like the total aerobic colony count and *Lactobacillus* spp. at To.

- Results of the plates made 24 hours after To (9h00-21/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	>/>	>/>	>/>	>/>	>/>	>/>	>/>	>/>
	-1/-1/-1	>/>	12/14	24/26	>/>	23/29	9/10	16/17	25/34
	-2/-2	27/29	1/NC	1/6	26/40	3/4	2/NC	2/3	5/6

Table 97: Results 24 hours after To at 15°C with N_2-flushing on M17

Log forming units on the plates after 3 days: (calculation same as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	5.1	3.8	4.1	5.2	4.1	3.7	3.9	4.2

Table 98: Log forming units 24 hours after To with N_2-flushing on M17

In table 97, results are noted of the colony counts of *Streptococcus* spp., made 24 hours after To, counted after 3 days. In table 98, the calculations of the log forming units are noted. There is growth of *Streptococcus* spp. in each condition after 24 hours at 15°C, with exception of condition HTN. This condition has a bacteriostatic effect on *Streptococcus* spp. after 24 hours at 15°C. In the other conditions, the lactoperoxidase-system lost his activity, but the N_2-flushing in this condition, inhibits the growth after 24 hours at 15°C. Condition HT and HN, show growth but the growth is very small, so these conditions are also effective to keep the log forming units low after 24 hours. That's also what's described in the first experiment, same conclusions can be made.

- Results of the plates made 48 hours after To (9h00-22/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	-1/-1/-1	>	>/>	>/>	>/>	>/>	71/67	>/>	>/>
	-2/-2/-2	>/>	25/15	>/>	>/>	>/>	4/14	30/33	>/>
	-3/-3/-3	16/17/31	4/2	20/22	25/26/36	18/12	1/1	7/4	17/12

Table 99: Results 48 hours after To at 15°C with N_2-flushing on M17

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	6.0	5.0	6.0	6.2	5.9	4.7	5.2	5.9

Table 100: Log forming units 48 hours after To with N_2-flushing on M17

In table 99, results are noted of the colony counts of *Streptococcus* spp. on M17 when the raw milk is kept 48 hours at 15°C. The log forming units are calculated and noted in table 100. There is growth in each condition. Conditions HTN and HT are most effective because of the lowest log forming units at this point, even there is growth. That's also what's described in the first experiment about *Streptococcus* spp.

- Results of the plates made 72 hours after To (9h00-23/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	-2/-2		>/>			>/>	>/>	>/>	>/>
	-3/-3	>	>/>	>/>	>	>/>	56/54	>/>	>/>
	-4/-4	>/>	37/38	>/>	>/>	49/54	15/15	28/37	72/62
	-5/-5	28/45		43/40	42/42				
	-6	8			3				

Table 101: Results 72 hours after To at 15°C with N_2-flushing on M17

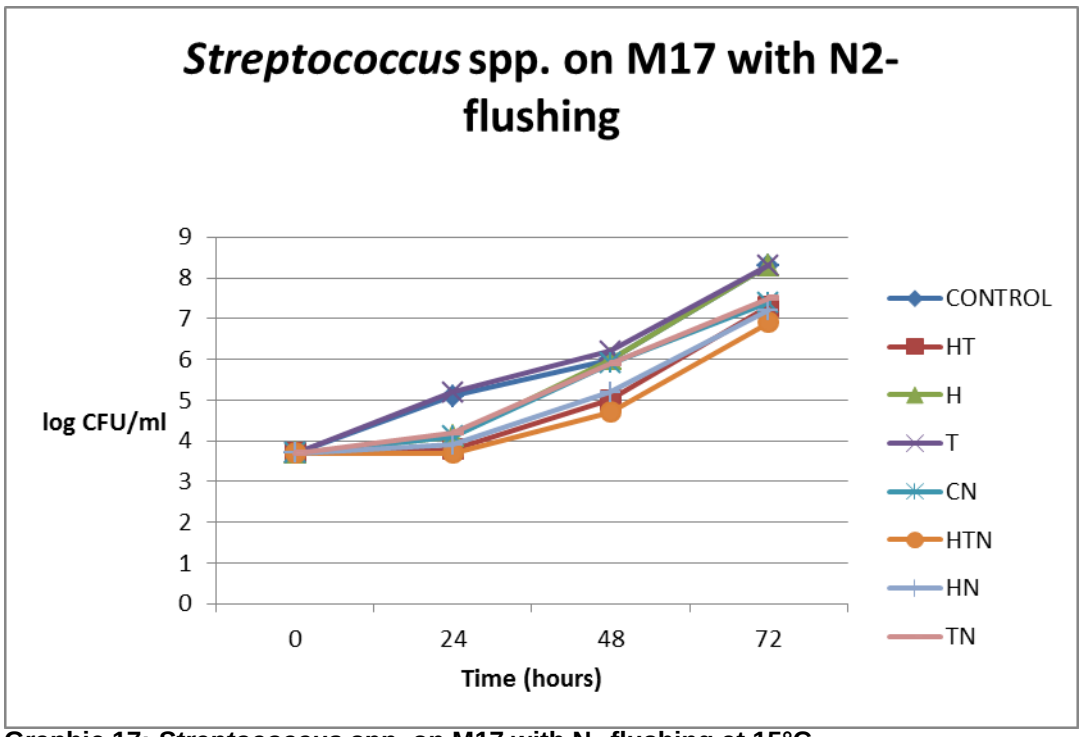
Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	8.3	7.3	8.3	8.3	7.4	6.9	7.2	7.5

Table 102: Log forming units 72 hours after To with N_2-flushing on M17

In tables 101 and 102, results can be found of the M17-plates made 72 hours after To at 15°C. After 72 hours at 15°C, same conclusion can be made as at 48 hours. There is growth of *Streptococcus* spp. but condition HTN keeps the log forming units lower than the other conditions. So here, condition HTN is the best condition, that's what's also concluded for the total aerobic colony count and *Lactobacillus* spp.

- Graphic



Graphic 17: *Streptococcus* spp. on M17 with N_2-flushing at 15°C

Graphic 17 shows results of the log forming units of *Streptococcus* spp. counted in raw milk kept at 15°C. There are different counting points at different moments. HTN is the best condition, with lowest log forming units, even there is growth after 48 hours.

4.7 Results of the first lactoperoxidase-test at 25°C with N_2-flushing

A first lactoperoxidase-test at 25°C with N_2-flushing was set up. In this test, the temperature is arisen up for 10 degrees, so following the literature study, certain effects will appear faster because of the higher temperature. There are eight conditions: CONTROL, HT, H, T, CN, HTN, HN and TN. Different counting points are made on PCA, MRS and M17.

4.7.1 Colony forming units on PCA (Plate Count Agar)

- Results of the plates made at To (8h00- 27/4/2015)

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 1	ND/ND/ND	?/?/19							
	-1/-1/-1	7/7/6							
DAY 2	ND/ND/ND	22/15/24							
	-1/-1/-1	7/7/7							
DAY 3	ND/ND/ND	24/25/24							
	-1/-1/-1	8/8/10							

Table 103: Results of To at 25°C with N_2-flushing

Log forming units of the plates after 3 days

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.6							

Table 104: Log forming units at To on PCA with N_2-flushing

In table 103, the results are noted down of the colony counts each day. Each day, there are little changes in counts. In table 104, the initial microflora of the total aerobic colony count is calculated for this first experiment at 25°C. There are 3.6 log forming units present in the raw milk at the start of the experiment. This is a moderate value, so the raw milk isn´t very fresh or it comes from different farms. But the value stays acceptable.

- Results of the plates made at T1 (11h00-27/4/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	-1/-1/-1	9/7/5	2/2/2	10/5/9	9/9/10	4/9/5	1/4/3	2/5/4	6/6/4
	-2/-2/-2	2/2/NC	1/NC/NC	2/1/1	NC/2/3	NC/NC/NC	NC/NC/NC	2/NC/NC	3/NC/NC
DAY 2	-1/-1/-1	9/7/11	7/3/2	13/7/11	14/9/10	7/13/8	4/5/4	3/6/8	7/8/6
	-2/-2/-2	2/3/NC	1/NC/NC	2/1/1	NC/2/3	3/NC/NC	NC/NC/NC	3/1/NC	4/NC/NC
DAY 3	-1/-1/-1	9/9/12	7/5/3	13/7/11	14/11/10	8/13/9	8/5/4	4/6/9	10/8/8
	-2/-2/-2	2/5/NC	1/NC/NC	3/2/3	NC/3/3	3/NC/NC	NC/NC/NC	4/2/9	4/NC/NC

Table 105: Results of T1 at 25°C on PCA with N_2-flushing

Log forming units of the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.7	3.4	3.7	3.8	3.7	3.5	3.5	3.6

Table 106: Log forming units at T1 with N_2-flushing

The results of the PCA-plates made 3 hours after To, are written in table 105. Each day, colonies are counted on the plates to see differences. Between day 1 and day 2, there are most differences in colony counts. There is not much growth between day 2 and day 3 during incubating at 30°C.

At T1, there is growth in conditions CONTROL, H, T and CN. This can be found in table 106. The growth is not big, but there are just 3 hours between To and T1. Condition HT, HTN and HN, show a decrease in log forming units when holding the raw milk 3 hours at 25°C. So these conditions are most effective at this counting point. In condition HT, the lactoperoxidase-system has a bactericidal effect on the total aerobic count. So the lactoperoxidase-system is only activated in this experiment when both substrates thiocyanate and H_2O_2 are added. There is no activation of the lactoperoxidase-system in the other conditions. In condition HN, it's the N_2-flushing that inhibits the growth of the total aerobic colony count, because condition H doesn't show a decrease in log forming units or activation of the lactoperoxidase-system. Condition TN shows a stable result after 3 hours, there is no decrease or increase of the log forming units of the total aerobic colony count. So this condition has a bacteriostatic effect. It's the N_2-flushing that inhibits the growth in this condition, because condition T doesn't show activation of the lactoperoxidase-system. All other conditions show growth.

- Results of the plates made at T2 (15u00-27/4/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	-1/-1		NC/NC	6/7		9	1/3	1	10
	-2/-2	8/5	NC/NC	1/NC	2/3	1/NC	2/NC	2/NC	NC/NC
	-3/-3/-3	1/NC	NC/NC	NC/NC	1/1	1/NC/NC	NC/NC	2/NC/NC	NC/NC/NC
	-4/-4	NC/NC			NC/NC				
DAY 2	-1/-1		2/2	11/9		11	1/ 4	1	17
	-2/-2	9/5	NC/NC	1/NC	2/3	1/NC	3/NC	2/NC	1/ 2
	-3/-3/-3	1/NC	NC/NC	NC/NC	1/1	1/NC/NC	NC/NC	2/NC/NC	NC/NC/NC
	-4/-4	NC/NC			NC/NC				
DAY 3	-1/-1		2/5	11/9		13	3/5	3	17
	-2/-2	10/5	1/NC	2/NC	2/3	1/1	3/NC	2/NC	1/ 2
	-3/-3/-3	1/NC	NC/NC	NC/NC	1/1	1/NC/NC	NC/NC	2/NC/NC	NC/NC/NC
	-4/-4	NC/NC			NC/NC				

Table 107: Results of T2 at 25°C on PCA with N_2-flushing

Log forming units of the plates after three days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	4.6	3.2	3.7	4.1	3.8	3.3	3.2	3.9

Table 108: Log forming units at T2 with N_2-flushing

In table 107, the results are noted of the colony counts after each day of incubation at T2. Between day 2 and day 3, there are almost no changes in colony counts. There are most differences in results between day 1 and day 2.

In table 108, the log forming units of the total aerobic colony counts are calculated for each condition. There is growth of the total aerobic colony count after 7 hours at 25°C, in conditions CONTROL, T, CN and TN, like noticed at T1. Just the bacteriostatic effect in condition TN is lost, and growth is already possible when holding the raw milk 7 hours at 25°C. Conditions HT, HTN and HN show a decrease in log forming units between T1 and T2. So the lactoperoxidase-system has still a bactericidal effect in these conditions and also N_2-flushing plays a role in inhibiting the growth of the total aerobic colony count. Condition H shows a stable result of the log forming units between T1 and T2, so the lactoperoxidase-effect has a bacteriostatic effect in this condition when just adding H_2O_2.

- Results of the plates made at T3 (18u00-27/4/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	-1/-1		2/3	23		10	1/NC	4/12	11
	-2/-2	108	NC/2	14/52	>	3/2	NC/NC	NC/3	2
	-3/-3	13	NC/NC	1/NC	12	NC/NC	NC/NC	1/NC	NC/NC
	-4/-4	2/NC		NC	1/3	NC			NC/NC
	-5/-5	NC/4			1/NC				
DAY 2	-1/-1		4/4	36		18	5/3	7/14	13
	-2/-2	>	2/2	36/>	>	5/4	1/NC	NC/3	2
	-3/-3	14	NC/NC	1/1	13	1/1	NC/NC	1/NC	NC/NC
	-4/-4	2/7		NC	1/3	1			NC/NC
	-5/-5	NC/5			1/1				

Table 109: Results of T3 at 25°C on PCA with N_2-flushing

Log forming units of the plates after three days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	5.8	3.3	4.3	5.8	4.4	3.3	3.7	3.8

Table 110: Log forming units at T3 with N_2-flushing

Table 109 shows the results of the colony counts each day of incubation at 30°C, of the point made 11 hours after To. The results of day 3 are identical to the results of day 2. There were no differences in colony counts. This is why the results of day 3 aren´t written in the table.

Table 110, shows the calculated log forming units of point T3. The only condition that shows a stable result is condition HTN. This condition seems to be most effective, still after 11 hours at 25°C. There is a bacteriostatic effect, so no growth of the total aerobic colony count is possible. The other conditions show growth of the total aerobic colony count, that´s quite normal following the literature study. When holding raw milk at 25°C, the lactoperoxidase-system is activated for 11-12 hours by adding one or both substrates. In this case, the activation is gone. It´s the N_2-flushing in condition HTN that inhibits the growth of the total aerobic colony count. Condition TN shows a decrease in log forming units between T2 and T3, so in this case there is a bactericidal effect. That's strange, because at T1 and T2, there

was growth. So the possible explanation here is faults in precision and diluting. Important to say, unless there is growth in condition HT, it´s still an effective condition to hold the total aerobic colony count low after 11 hours, just like condition HTN. Conditions HT and HTN show lowest log forming units at this counting point, so here they are most effective.

- Results of the plates made 24 hours after To (8h00-28/4/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	-1/-1		>/>			>/>	>/>	>/>	>/>
	-2/-2		>/>	>		>/>	85/88	>/>	>/>
	-3/-3	>	>/>	>/>	>	>/>	12/19	>/>	>/>
	-4/-4	>/>		>/>	>/>				
	-5/-5/-5	>/>	5/7/1	124	>/>	30/33/36		5/5/8	3/9/12
	-6/-6/-6/-6	30/27/25/32		13/23/24	32/16/10/15				
DAY 2 and 3	-1/-1		>/>			>/>	>/>	>/>	>/>
	-2/-2		>/>	>		>/>	85/88	>/>	>/>
	-3/-3	>/>	>/>	>/>	>/>	>/>	12/19	>/>	>/>
	-4/-4	>/>		>/>	>/>				
	-5/-5/-5	>/>	5/7/1	124	>/>	30/33/36		5/5/8	3/9/12
	-6/-6/-6/-6	30/27/25/32		13/23/24	32/16/10/15				

Table 111: Results 24 hours after To at 25°C on PCA with N_2-flushing

Log forming units of the plates after three days: (same calculation as written above)

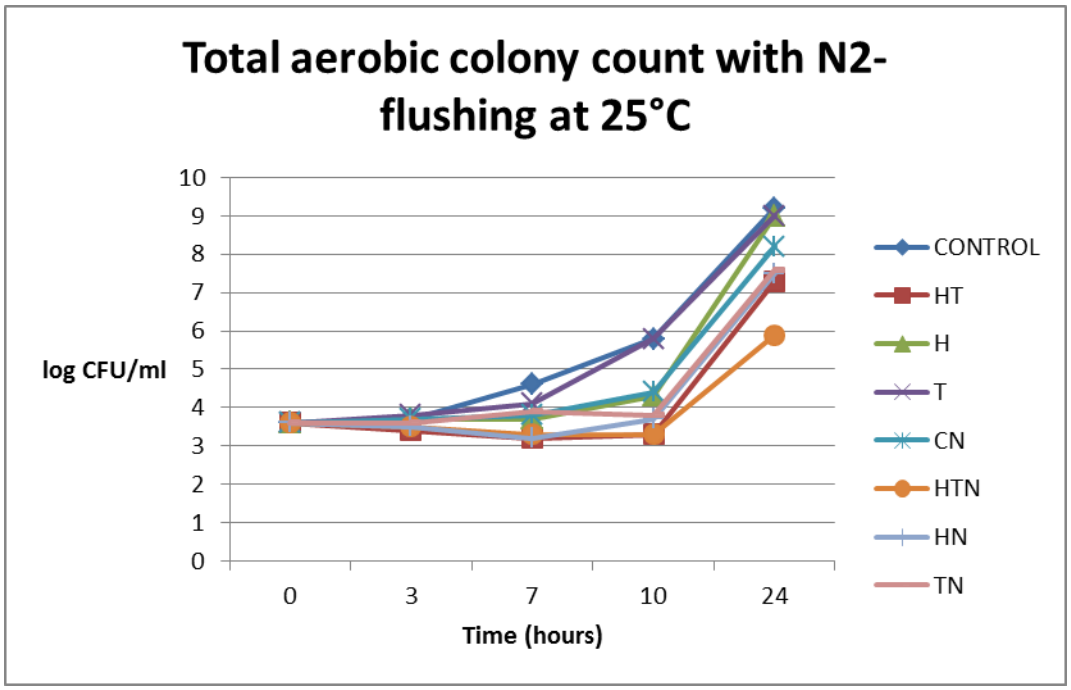
	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	9.2	7.3	9.0	9.0	8.2	5.9	7.5	7.6

Table 112: Log forming units 24 hours after To with N_2-flushing

Table 111 shows the results of the colony counts each day of incubation at 30°C. There are no differences in the total aerobic colony counts between day 1 and day 2, but there are even differences between day 2 and day 3. That´s the reason why the counts weren´t repeat in the table for day 3. So the counts at day 1 stay identical until the third day of incubation at 30°C.

In table 112, the log forming units were calculated and noted down, when the raw milk is kept 24 hours at 25°C. There is growth in each condition, what´s quite normal following the literature study. Unless there is growth, conditions HT and HTN still show the lowest total aerobic colony counts. Condition HTN shows lowest log forming units when holding raw milk 24 hours at 25°C, what means N_2-flushing has and inhibition effect. It´s the best condition, even after 24 hours.

- Graphic



Graphic 18: Total aerobic colony count with N_2-flushing on PCA at 25°C

Graphic 18 shows the comparison between the different total aerobic colony counts of the eight conditions at different counting points. After 3 hours at 25°C, there are not lots of differences in counts, as can be seen on the graphic. Log forming units are lying near to each other. Conditions HT, HTN and HN show a light decrease. After 7 hours, there are already more differences, the log forming units are more spread. Even at this counting point, conditions HT, HTN and HN show the lowest log forming units, and there is still decrease of the total aerobic colony count in these conditions. The other conditions show light growth. After 10 hours at 25°C, results are more spread than after 7 hours, but conditions HT, HTN and HN, are lying near to each other, Log forming units don´t differ a lot. Condition HTN is the only stable condition. After 24 hours, there is growth of the total aerobic colony count in each condition. This is also shown on the graphic.

4.7.2 Colony forming units on MRS

- Results of the plates made at To (8h00 – 27/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	>/>/>							
	-1/-1/-1	4/4/2							

Table 113: Results of To at 25°C on MRS with N_2-flushing

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.2							

Table 114: Log forming units at To with N_2-flushing on MRS

Tables 113 and 114 show the initial microflora of *Lactobacillus* spp. at To. The value of 3.2 log forming units is moderate. Same conclusion can be made as on PCA, the raw milk isn't very fresh. There is already a lot of contamination but the value stays acceptable.

- Results of the plates made at T3 (18h00-27/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND		17/27	>/>		>/>	20/22	26/13	>/>
	-1/-1	40/41	2/3	26/23	50/51	7/16	7/3	5/7	9/12
	-2/-2	7/6	2/NC	2/1	8/8	1/3	1/NC	NC/NC	1/NC
	-3/-3	2/2			1/1				

Table 115: Results of T3 at 25°C with N_2-flushing on MRS

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	4.3	3.0	4.1	4.4	3.8	3.0	3.0	3.7

Table 116: : Log forming units of T3 with N_2-flushing on MRS

The results of the colony counts of *Lactobacillus* spp. after 3 days of incubation at 30°C are noted down in table 115, when raw milk is kept 10 hours at 25°C.

In table 116, the log forming units are calculated for each condition. Conditions HT, HTN and HN are the only conditions that show a decrease of the log forming units after 10 hours. So these conditions are the only conditions that seem to be effective. In the literature study it´s written that the lactoperoxidase-system is activated for 11-12 hours when raw milk is kept at 25°C. Here, the lactoperoxidase-system isn´t activated in the other conditions. In condition HT, there is a bactericidal effect by activation of the lactoperoxidase-system. Conditions HTN and HN show bactericidal effects by activation of the lactoperoxidase-system and N_2-flushing.

Results don't differ a lot with the results of PCA, but there are still differences. Here the lactoperoxidase-system is still activated in condition HT, because there is a bactericidal effect on *Lactobacillus* spp., so the growth on PCA, means there was unpunctual working. In condition HTN, the lactoperoxidase-system is also activated and N_2-flushing inhibits growth. Condition HN also shows a decrease in log forming units, what means only adding H_2O_2 and N_2-flushing has a bactericidal effect on *Lactobacillus* spp. after 10 hours at 25°C. On PCA, this condition also showed first decreases but not up to 10 hours after To, so on MRS, this condition has a longer effect than on PCA. All other conditions show growth, so no lactoperoxidase-system is activated or N_2-flushing has no inhibiting effect in the other conditions.

- Results of the plates made 24 hours after To (8h00-28/4/2015) AFTER 3 DAYS

	Dilution	**CONTROL**	**HT**	**H**	**T**	**CN**	**HTN**	**HN**	**TN**
DAY 3	-1/-1		>/>	>/>		>/>	>/>	>/>	>/>
	-2/-2	>/>	>/>	>/>	>/>	>/>	>/82	>/>	>/>
	-3/-3	>/>	156/108	>(*)/236	>/>	312(*)/>	17/24	117/114	94/92
	-4/-4	270 (*)/>			176(*)/>				

Table 117: Results 24 hours after To at 25°C with N_2-flushing on MRS

(*): excessive counts under estimation (1 value)

Log forming units on the plates after 3 days: (same calculation as written above)

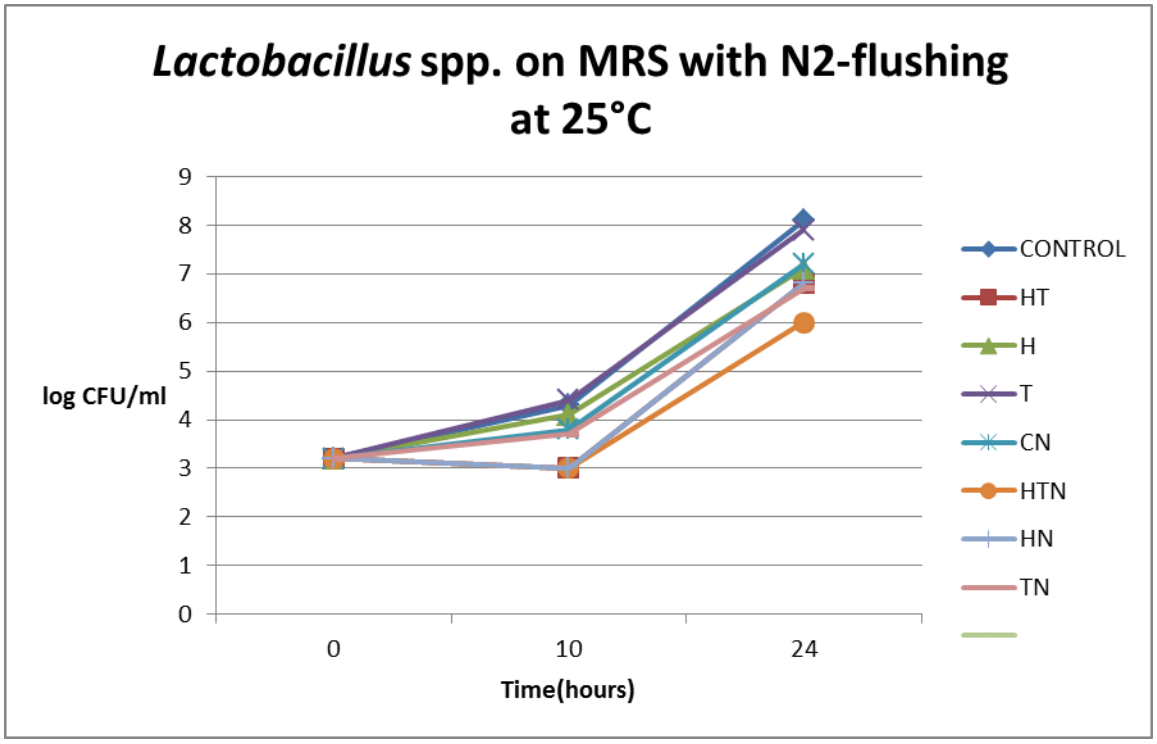
	CONTROL	**HT**	**H**	**T**	**CN**	**HTN**	**HN**	**TN**
Log forming units after 3 days	8.1	6.8	7.1	7.9	7.2	6.0	6.8	6.7

Table 118: Log forming units 24 hours after To with N_2-flushing on MRS

In table 117, the results of the counts of *Lactobacillus* spp. after 3 days at 30°C are noted down. At this point, the raw milk is kept 24 hours at 25°C. There is excessive growth in most conditions, so it was difficult to count the plates precisely.

In table 118, the log forming units are calculated and they show growth of *Lactobacillus* spp. in each condition. It´s normal the lactoperoxidase-system has no more activation after 24 hours at 25°C. Unless there is growth, condition HTN shows the lowest colony counts, so even after 24 hours this condition is most effective with lowest growth, identical as described on PCA. So N_2-flushing has a positive inhibiting effect when it´s added. Holding raw milk 24 hours at 25°C, is no option to hold the log forming units of *Lactobacillus* spp. low..

- Graphic



Graphic 19: *Lactobacillus* spp. on MRS with N_2-flushing at 25°C

When there are 3.2 log forming units at To, there is a decrease of *Lactobacillus* spp. after 10 hours at 25°C in conditions HT, HTN and HN. This is also shown on the graphic. The other conditions show growth after 10 hours on graphic 19, but results are not spread at all. There are not lots of differences in counts at this point. After 24 hours at 25°C, the results of the different conditions stay near to each other. They arent´spread. But there is growth in each condition.

4.7.3 Colony forming units on M17

- Results of the plates made at To (8h00 – 27/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	20/19/18							
	-1/-1/-1	1/9/10							

Table 119: Results of To at 25°C with N_2-flushing on M17

Log forming units on the plates after 3 days: (calculation same as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.0							

Table 120: Log forming units at To with N_2-flushing on M17

Table 119 and 120 show the initial microflora of *Streptococcus* spp. on M17 after 3 days of incubation at 30°C. This is a moderate value, but there is already a lot of contamination at To, so the raw milk isn´t very fresh.

- Results of the plates made at T3 (18h00-27/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND		15/17	>/>		>/>	15/19	>/>	>/>
	-1/-1	34/37	8/7	23/29	47/50	23/26	5/3	7/13	9/5
	-2/-2	9/4	1/NC	3/6	5/9	3/1	NC/NC	27/NC	1/NC
	-3/-3	3/2			3/NC				

Table 121: Results of T3 at 25°C with N_2-flushing on M17

Log forming units on the plates after 3 days: (calculation same as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	4.2	2.9	4.1	4.4	4.1	2.9	3.7	3.5

Table 122: Results of T3 at 25°C with N_2-flushing on M17

Table 121 shows the colony counts of *Streptococcus* spp. after 3 days of incubation at 30°C, when holding the raw milk 10 hours at 25°C.

Table 122 shows the calculated log forming units. Conditions HT and HTN are the only conditions which show a decrease in log forming units after 10 hours at 25°C. That's also what's described on PCA and MRS. So these conditions have a bactericidal effect on *Streptococcus* spp. at this condition. This is strange, normally the lactoperoxidase-system should be activated in the other conditions, but that´s not what´s going on here. The lactoperoxidase-system doesn´t show any effect on *Streptococcus* spp. in the other

conditions. Only when adding both substrates, the lactoperoxidase-system has a bactericidal effect in this situation. Adding both substrates and N_2-flushing shows the same result as condition HT.

- Results of the plates made 24 hours after To (8h00-28/4/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	-1/-1		>/>	>/>		>/>	>/>	>/>	>/>
	-2/-2	>/>	>/>	>/>	>/>	>/>	>/>	>/>	>/>
	-3/-3	>/>	74/76	>/>	>/>	100(*)/>	9/16	72/64	54/58
	-4/-4	100(*)/>		98(*)	127(*)/>				

Table 123: Results 24 hours after To at 25°C with N_2-flushing on M17

(*): excessive counts under estimation (1 value)

Log forming units on the plates after 3 days: (calculation same as written above)

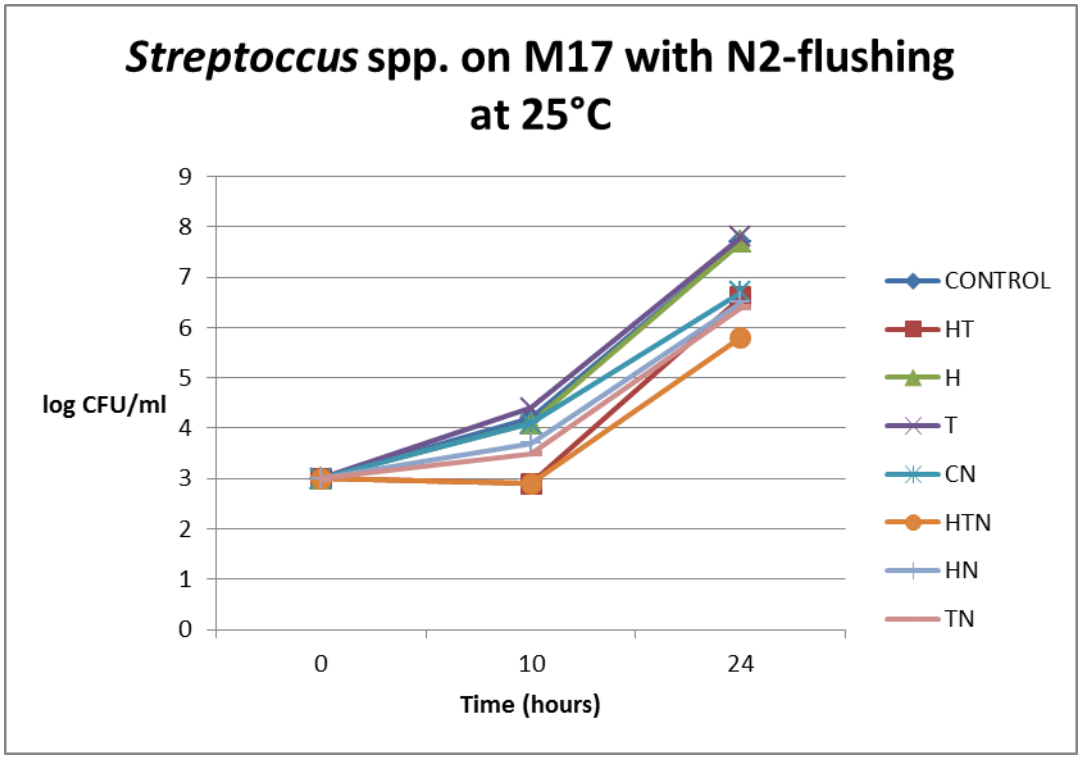
	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	7.7	6.6	7.7	7.8	6.7	5.8	6.5	6.4

Table 124: Log forming units 24 hours after To with N_2-flushing on M17

Table 123 shows excessive growth of *Streptococcus* spp. in a lot of conditions after holding raw milk 24 hours at 25°C. That´s normal, because there is no more activation of the lactoperoxidase-system after 24 hours at 25°C. So it´s just N_2-flushing that can hold the growth of *Streptococcus* spp. low.

In table 124, the log forming units are calculated. Condition HTN shows the lowest result, what means, even there is growth after 24 hours, condition HTN seems to be most effective to hold the colony counts of *Streptococcus* spp. lower than the other conditions.

- Graphic



Graphic 20: *Streptococcus* spp. on M17 with N_2-flushing at 25°C

Graphic 20 shows the results of the growth of *Streptococcus* spp. in the different conditions. After holding raw milk 10 hours at 25°C, results don´t show lots of differences on the graphic. Results aren´t spread. Conditions HT and HTN are the only conditions that show a decrease in log forming units. That´s also shown on the graphic. After 24 hours, there is growth in each condition on the graphic, but condition HTN shows the lowest log forming units of *Streptococcus* spp.

4.8 Results of the second lactoperoxidase-test at 25°C with N_2-flushing

A second experiment is set up at 25°C with N_2-flusing to confirm the results and statements made in the first experiment written above. In this experiment, there are again eight conditions. Of these eight conditions, counting points are made at To (20h30-04/05/2015), 12 hours after To (T1), 17 hours after To (T2) and 24 hours after To. Counting points are made on PCA, MRS and M17.

4.8.1 Colony forming units on PCA (Plate Count Agar)

- Results of the plates made at 9h00- 04/05/2015

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 1	ND/ND/ND	12/13/>							
	-1/-1/-1	8/7/1							
DAY 2	ND/ND/ND	23/20/>							
	-1/-1/-1	9/8/2							
DAY 3	ND/ND/ND	31/26/>							
	-1/-1/-1	9/12/3							

Table 125: Results of the controlling point at 25°C with N_2-flushing

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.2							

Table 126: Log forming units of the controlling point on PCA with N_2-flushing

This counting point on PCA is a controlling point. It´s NOT To. This plate is made to detect differences in colony counts between this point and To made at 20h30 in the evening (04/05/2015).

After each day of incubation, there is growth of the total aerobic colony count, this can be seen in table 125, where the counts are changing every day. In table 126, the log forming units of the total aerobic colony count of the counting point at 9h00- 04/05/2015 are written. This is a moderate value, but acceptable. The raw milk is not very fresh.

- Results of the plates made at To (20h30- 04/05/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	ND/ND/ND	NC/NC/NC							
	-1/-1/-1	NC/NC/NC							
DAY 2	ND/ND/ND	12/19/21							
	-1/-1/-1	3/3/5							
DAY 3	ND/ND/ND	13/23/23							
	-1/-1/-1	5/3/6							

Table 127: Results of To at 25°C with N_2-flushing

Log forming units of the plates after 3 days

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.0							

Table 128: Log forming units at To on PCA with N_2-flushing

The results of the colony counts at To are written in table 127. After each day of incubation, there is growth of the total aerobic colony counts on the PCA-plates. There are changes in counts. In table 128, the initial microflora of the total aerobic colony count at To is calculated. There are 3.0 log forming units present, what means the raw milk isn´t very fresh. That´s also described at the point at 9h00-04/05/2015. The values of the log forming units at To and the point at 9h00 don´t differ a lot, so that´s positive. This means there is no growth of the total aerobic colony count between the morning and the evening point at To.

- Results of the plates made at T1 (8h30- 05/05/2015) = 12 hours after To

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	-1/-1		11/17	>/>		126/129	8/8	55/67	>/>
	-2/-2	>	NC/NC	>/>	>	16/25	1/NC	7/7	32/51
	-3/-3/-3	>/>/>	NC/NC	55/70	>/>/>	3/6	NC/NC	1/NC	6/8
	-4/-4	16/27			11/19				
DAY 2	-1/-1		11/18	>/>		126/129	9/8	57/68	>/>
	-2/-2	>	1/1	>/>	>	17/25	1/1	7/7	32/51
	-3/-3/-3	>/>/>	NC/NC	55/70	>/>	3/6	NC/NC	1/NC	6/8
	-4/-4	27/17			11/19				
DAY 3	-1/-1		12/18	>/>		126/129	10/8	57/68	>/>
	-2/-2	>	1/1	>/>	>	17/25	1/1	10/9	32/51
	-3/-3/-3	>/>/>	1/NC	55/70	>/>/>	3/6	NC/NC	1/NC	6/9
	-4/-4	16/27			11/19				

Table 129: Results of T1 at 25°C with N_2-flushing on PCA

Log forming units of the plates after 3 days

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	7.0	3.9	6.5	6.9	5.0	3.7	4.7	5.6

Table 130: Log forming units at T1 on PCA with N_2-flushing

In table 129, the results are noted of the colony counts of the PCA-plates made 12 hours after To (20h30-04/05/2015). After each day of incubation at 30°C, the total aerobic colony counts were count. Each day, there is growth, so there are differences between day 1 and day 2, but also between day 2 and day 3. Normally, colony counts don't change a lot between day 2 and day 3. In this case, it's contrary.

In table 130, the log forming units of the eight conditions are calculated after 3 days of incubation at 30°C. There is growth of the total aerobic colony count in each condition. That's normal, because the lactoperoxidase-system has an activation of 11-12 hours, when the raw milk is kept at 25°C. This is written in the literature study. So, there is no more activation of the lactoperoxidase-system at T1. Activation is gone. Unless there is growth in each condition, conditions HT and HTN show lowest growth, what stays positive. The growth isn't big. The conditions with N_2-flushing also show lower growth than the other conditions without N_2-flushing.

- Results of the plates made at T2 (15h30- 05/05/2015) = 17 hours after To

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	-1/-1		>			>	>	>	>
	-2/-2		48/60			>	13	69	>
	-3/-3	>/>	6/10	>/>	>/>	13/17	1/1	9/7	>/>
	-4/-4	>/>	2	52/63	>/>	NC/NC	1	1/NC	9/12
	-5/-5	15/17		8/10	12/10				
DAY 2 and 3	-1/-1		>			>	>	>	>
	-2/-2		49/60			>	13	69	>
	-3/-3	>/>	7/10	>/>	>/>	13/17	1/1	9/7	>/>
	-4/-4	>/>	2	52/63	>/>	1/1+1	1	1/NC	9/12
	-5/-5	15/17		8/10	12/10				

Table 131: Results of T2 at 25°C with N_2-flushing on PCA

Log forming units of the plates after 3 days

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	7.9	5.6	7.7	7.7	5.9	4.8	5.6	6.7

Table 132: Log forming units at T2 on PCA with N_2-flushing

In table 131, the results are noted of the colony counts after each day of incubation, of the plates made 17 hours after To (20h30-04/05/2015). Between day 1 and day 2, there is growth of the total aerobic colony count in most conditions. Between day 2 and day 3 of incubation at 30°C, there are no more changes or there is no more growth, so the results are the same. That´s why the results aren´t copied. They are written together.

In table 132, the log forming units are calculated of the PCA-plates made at T2. There is growth in each condition. That's logical, because there was already growth at T1. Same statements can be made as at T1. Conditions HT and HTN show lowest growth and the conditions with N_2-flushing show lower results of the total aerobic colony counts than the other conditions. So conditions HT and HTN are still most effective.

- Results of the plates made 24 hours after To (20h30-05/05/2015)

DAY 1	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
	-1/-1						>		
	-2/-2						>		
	-3/-3		>				>/>		
	-4/-4/-4		16/16/22			>/>	12/14	>/>	>/>
	-5/-5	77	3/4	>	95	22/14		13/14	50/62
	-6/-6/-6	10/10/8		10/5/8	NC/5/7	4/NC		2/NC	9/6
	-7/-7	NC/NC		2/NC	NC/NC				
DAY 2	-1/-1						>		
	-2/-2						>		
	-3/-3		>				>/>		
	-4/-4/-4		16/16/22			>/>	12/14	>/>	>/>

	-5/-5	77	3/4	>	96	22/15		13/15	50/62
	-6/-6/-6	12/10/8		10/5/8	NC/6/7	4/NC		3/NC	10/6
	-7/-7	NC/NC		2/NC	1/NC				
DAY 3	-1/-1						>		
	-2/-2						>		
	-3/-3		>				>/>		
	-4/-4/-4		16/16/22			>/>	12/14	>/>	>/>
	-5/-5	77	3/4	>	96	22/15		13/15	50/62
	-6/-6/-6	12/13/8		10/5/9	NC/7/7	4/1		3/NC	10/6
	-7/-7	NC/NC		2/NC	1/NC				

Table 133: Results 24 hours after To at 25°C on PCA with N_2-flushing

Log forming units of the plates after three days: (same calculation as written above)

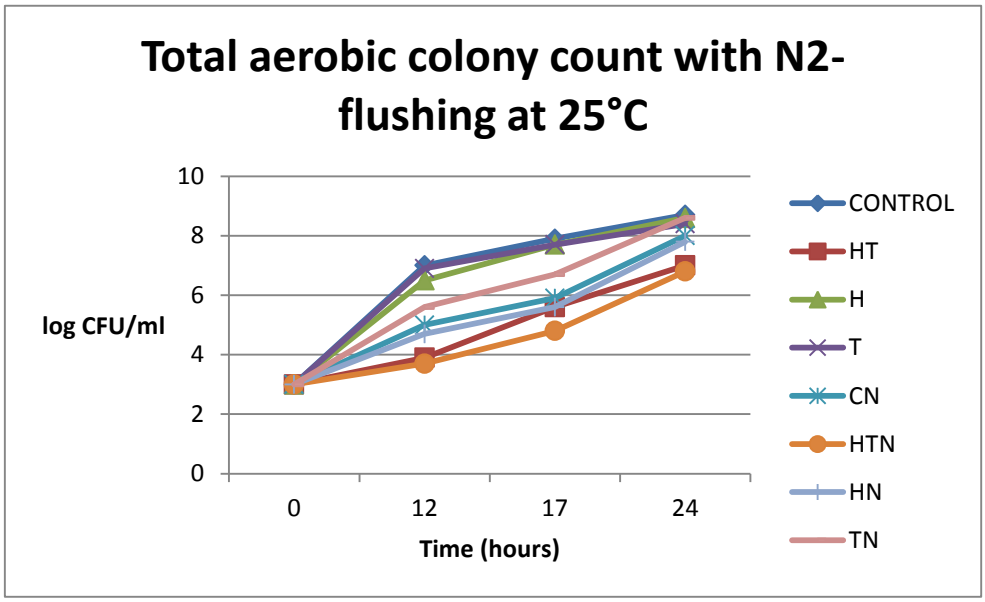
	CONTROL	**HT**	**H**	**T**	**CN**	**HTN**	**HN**	**TN**
Log forming units after 3 days	8.7	7.0	8.6	8.4	8.0	6.8	7.8	8.6

Table 134: Log forming units 24 hours after To with N_2-flushing

In table 133, results are noted of the colony counts after each day of incubation, of the plates made 24 hours after To (20h30-04/05/2015). Between day 1 and day 2, there is most growth of the total aerobic colony counts, changes are noted in the table. Between day 2 and day 3, there is not lot of growth of the total aerobic colony counts. Just a few conditions, show growth between day 2 and day 3 of incubation at 30°C.

The log forming units are calculated in table 134. Growth keeps going on. Same statements can be made as at T2. There is no changing trend.

- Graphic



Graphic 21: Total aerobic colony count with N_2-flushing on PCA at 25°C

Graphic 21 shows the comparison between growth of the total aerobic colony counts in the eight conditions at different counting points. After 12 hours, there is growth in each condition, and this growth keeps going on at each counting point. The growth is smallest in conditions HT and HTN. This is also showed on the graphic. The red and orange line show lowest log CFU/ml at each counting point. The lines of conditions CONTROL, H and T, show largest log CFU/ml at each counting point. That's also said above.

4.8.2 Colony forming units on MRS

- Results of the plates made at To (20h30- 04/05/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	14/8/8							
	-1/-1/-1	2/2/2							

Table 135: Results of To at 25°C with N_2-flushing

Log forming units of the plates after 3 days

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	2.7							

Table 136: Log forming units at To on MRS with N_2-flushing

In table 135, the results are written of the colony counts of *Lactobacillus* spp. at To after 3 days of incubation at 30°C. In table 136, the log forming units are calculated, what results in 2.7 log forming units of *Lactobacillus* spp. at the beginning of the experiment. There is not much contamination of the raw milk with this species. The initial microflora of *Lactobacillus* spp. is rather low.

- Results of the plates made at T1(8h30-05/05/2015) = made 12 hours after To AFTER 3 DAYS

	Dilution	**CONTROL**	**HT**	**H**	**T**	**CN**	**HTN**	**HN**	**TN**
DAY 3	ND/ND		>/>	>/>			>/>	>/>	>/>
	-1/-1	>/>	21/21	>/>	>/>	82/>	10/9	28/42	>/>
	-2/-2	44/49	1/NC	39/36	53/39	18/9	1/1	5/4	36/56
	-3/-3	5/8			2/3	1/4			

Table 137: Results of T1 at 25°C with N_2-flushing on MRS

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	**HT**	**H**	**T**	**CN**	**HTN**	**HN**	**TN**
Log forming units after 3 days	5.4	4.0	5.3	5.4	4.8	3.7	4.2	5.4

Table 138: Log forming of T1 with N_2-flushing on MRS

Table 137 shows the colony counts of *Lactobacillus* spp. on MRS at T1, made 12 hours after To (20h30-04/05/2015). After 3 days of incubation at 30°C, log forming units are calculated in table 138. There is growth of *Lactobacillus* spp. in each condition. That's normal, because there is no more activation of the lactoperoxidase-system, following the literature study. Same statements can be made as described in the part of the total aerobic colony count. Conditions HT and HTN show lowest growth, what means these are the most effective conditions to keep the growth lower. The conditions with N_2-flushing also show lower growth than the conditions without N_2-flushing.

- Results of the plates made at T2(15h30-05/05/2015) = made 17 hours after To AFTER 3 DAYS

	Dilution	**CONTROL**	**HT**	**H**	**T**	**CN**	**HTN**	**HN**	**TN**
DAY 3	-1/-1		>			>	65	>	>
	-2/-2		20/57			>	10	36	>

	-3/-3	>/>	7/8	>/>	>/>	15/10	2/NC	6/3	59/57
	-4/-4	11/15	NC	18/17	7/10	2/NC	1/NC	1/1	6/3
	-5/-5	4/NC		1/3	1/NC				

Table 139: Results of T2 at 25°C with N_2-flushing on MRS

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	6.8	5.6	6.9	6.6	5.8	4.7	5.4	6.5

Table 140: Log forming of T2 with N_2-flushing on MRS

Table 139 shows the results of the colony counts of *Lactobacillus* spp. on MRS, 17 hours after To (20h30-04/05/2015). In table 140, log forming units are calculated. Growth keeps going on. Same statements can be made as described at T1. So they won't be repeat.

- Results of the plates made 24 hours after To (20h30-04/05/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	-3/-3		>				31/34/31		
	-4/-4	>	15/8/12	>	>	80/72	5	27/30	>/>
	-5/-5/-5	34/35/31	2/2	43/46/53	55/44/47	6/13		4/3	42/44
	-6/-6	6/7		5/5	9/6	NC/NC		NC/NC	3/5

Table 141: Results 24 hours after To at 25°C with N_2-flushing on MRS

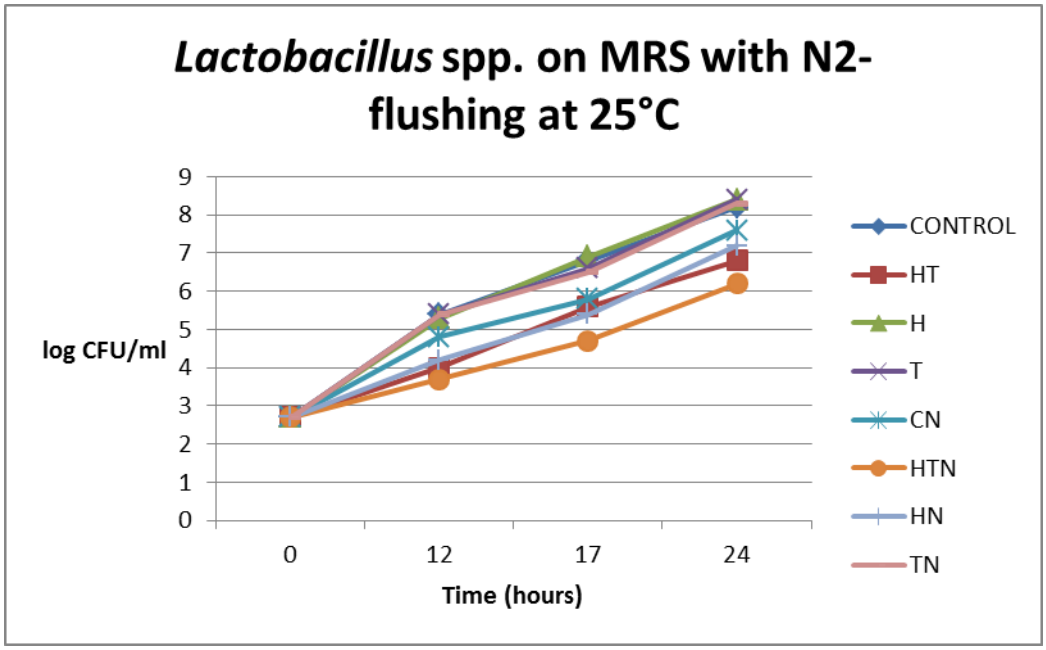
Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	8.2	6.8	8.4	8.4	7.6	6.2	7.2	8.3

Table 142: Log forming units 24 hours after To with N_2-flushing on MRS

Table 141 shows the colony counts of *Lactobacillus* spp. on MRS, 24 hours after To (20h30- 04/05/2015). In table 142, the accompanying log forming units are noted. Same statements can be made as at T1 and T2.

- Graphic



Graphic 22: *Lactobacillus* spp. on MRS with N_2-flushing at 25°C

Graphic 22 shows the comparison in growth between the eight conditions at different counting points. Same statements can be made as on PCA. There is growth after 12 hours and this growth of *Lactobacillus* spp. keeps going on until 24 hours after To. Conditions HT and HTN show lowest growth of *Lactobacillus* spp., what means, here on MRS, these are the most effective conditions to hold the growth lower than the other conditions. That's also what's told on PCA. The conditions with N_2-flushing are also more effective than the conditions CONTROL, HN and TN. Log CFU/ml of the different conditions are lying near to each other, so there are not much differences in colony counts between the different conditions.

4.8.3 Colony forming units on M17

- Results of the plates made at To (20h30- 04/05/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND/ND	21/21/22							
	-1/-1/-1	1/1/6							

Table 143: Results of To at 25°C with N_2-flushing

Log forming units of the plates after 3 days

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	3.0							

Table 144: Log forming units at To on M17 with N_2-flushing

In tables 143 and 144, results are written of the colony counts of *Streptococcus* spp. after 3 days of incubation at 30°C. The initial microflora of *Streptococcus* spp. in this experiment is 3.0 log forming units. This value is not high, it´s a moderate value.

- Results of the plates made at T1(8h30-05/05/2015) = made 12 hours after To AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	ND/ND		>/>	>/>			>/>	>/>	>/>
	-1/-1	>/>	4/17	>/>	>/>	>/>	18/7	37/35	>/>
	-2/-2	>/>	1/3	30/32	31/29	18/25	3/2	6/6	44/43
	-3/-3	7/13			4/8	3/NC			

Table 145: Results of T1 at 25°C with N_2-flushing on M17

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	5.7	3.7	5.2	5.2	5.0	3.8	4.3	5.3

Table 146: Log forming of T1 with N_2-flushing on M17

The results of the colony counts of *Streptococcus* spp. on M17, made 12 hours after To (20h30-04/05/2015), are noted in table 145 after 3 days of incubation at 30°C. Of these results at T1, log forming units are calculated in table 146. There is growth in each condition, because of the ended activation of the lactoperoxidase-system. Unless there is growth, conditions HT and HTN, keep the growth of *Streptococcus* spp. lowest, so these conditions are most effective after 12 hours for *Streptococcus* spp. That's identical for PCA and MRS at this counting point. The conditions with N_2-flushing are also more effective than without N_2-flushing.

- Results of the plates made at T2(15h30-05/05/2015) = made 17 hours after To AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	-1/-1		>			>	>	>	>
	-2/-2		31/32			>	11	>	>
	-3/-3	>/>	4/8	>/>	>/>	4/9	1/1	5/8	>/>
	-4/-4	12/19	NC	14/18	15/12	3/NC	1/NC	3/NC	9/8
	-5/-5	1/1		1/2	2/3				

Table 147: Results of T2 at 25°C with N_2-flushing on M17

Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	6.9	5.2	6.9	6.8	5.5	4.7	5.5	6.6

Table 148: Log forming of T2 with N_2-flushing on M17

The results of the colony counts of *Streptococcus* spp. on M17, made 17 hours after To (20h30-04/05/2015) are written in table 147. Table 148 shows the calculated log forming units at this counting point. There is still growth of *Streptococcus* spp. Same statements can be made as at T1. So they won't be repeat.

- Results of the plates made 24 hours after To (20h30-04/05/2015) AFTER 3 DAYS

	Dilution	CONTROL	HT	H	T	CN	HTN	HN	TN
DAY 3	-3/-3		>				27/26/34		
	-4/-4	>	7/12/12	>	>	>/>	5	51/54	>/>
	-5/-5/-5	31/24/32	3/NC	40/34/41	35/32/29	8/14		6/6	39/38
	-6/-6	8/3		1/5	8/4	2/1		NC/NC	9/6

Table 149: Results 24 hours after To at 25°C with N_2-flushing on M17

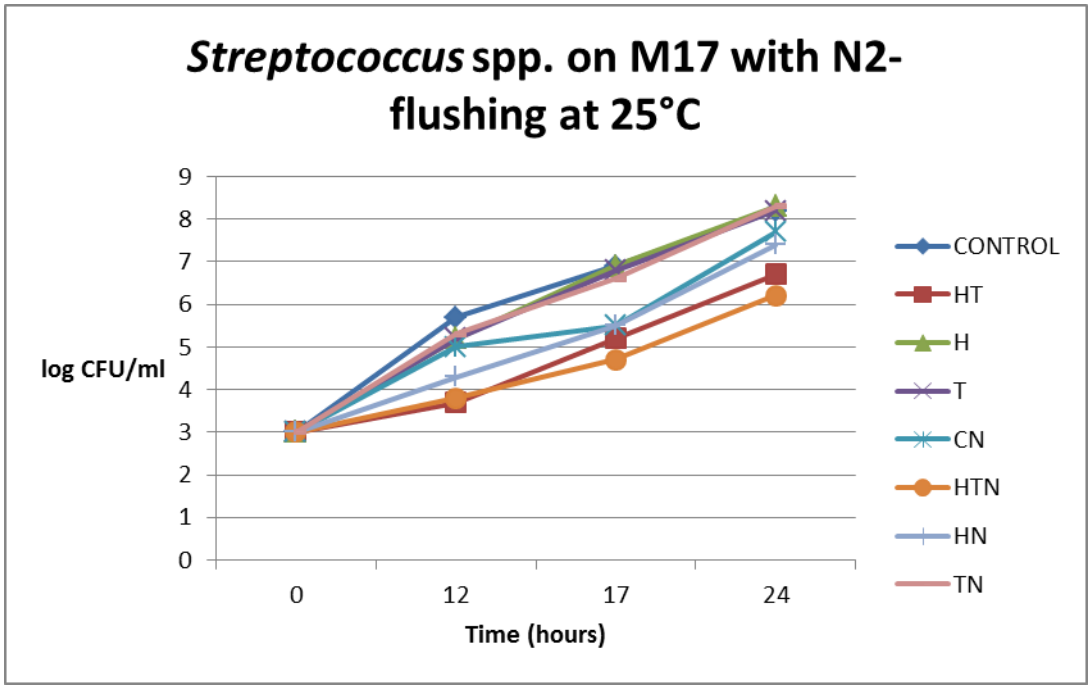
Log forming units on the plates after 3 days: (same calculation as written above)

	CONTROL	HT	H	T	CN	HTN	HN	TN
Log forming units after 3 days	8.2	6.7	8.3	8.2	7.7	6.2	7.4	8.3

Table 150: Log forming units 24 hours after To with N_2-flushing on M17

Table 149 shows the results of the colony counts of *Streptococcus* spp. on M17, made 24 hours after To. Of these results, log forming units are calculated in table 150. Growth keeps going on in each condition. Same statements can be made as at T1 and T2 for *Streptococcus* spp.

- Graphic



Graphic 23: *Streptococcus* spp. on M17 with N_2-flushing at 25°C

Graphic 23 shows the growth of *Streptococcus* spp. in each condition at different counting points. 12 hours after To, there is already growth in each condition of *Streptococcus* spp. and this growth keeps going on. Unless there is growth, conditions HT and HTN still show lower log CFU/ml than the other conditions. So these two conditions are most effective for *Streptococcus* spp. at each counting point. They hold the colony counts lower. What also can be concluded after looking to the graphic is the largest growth of *Streptococcus* spp. in conditions CONTROL, H and T. These conditions, where just one substrate is added and no N_2-flushing is added, show most log CFU/ml in each condition. So these conditions are not effective at all.

5 Conclusion

During the different experiments done at the lab, the effectivity was controlled of eight conditions.
These are the eight different conditions tested and examined at the lab:

- CONTROL: only raw milk, without any substrate or N_2-flushing added.
- HT: both substrates are added to the raw milk, thiocyanate and H_2O_2.
- H: only H_2O_2 is added to the raw milk.
- T: only thiocyanate is added to the raw milk.
- CN: N_2-flushing in the CONTROL-bottle.
- HTN: both substrates are added, thiocyanate and H_2O_2, but also N_2-flushing happens.
- HN: H_2O_2 is added and N_2-flushing happens.
- TN: thiocyanate is added and N_2-flushing happens.

For the experiments at 15°C and 25°C, where the effectivity and influence of the lactoperoxidase-system and N_2-flushing on the raw milk microflora was controlled and examined, same statements and descriptions were made in the part results. Of all the experiments done at the lab, same global conclusion can be made.

The lactoperoxidase-system is activated for maximum 24 hours at 15°C. When holding the raw milk at 25°C, the lactoperoxidase-system is activated for maximum 12 hours. That's also what's written in the literature study (FAO,2005). So the experiments confirm the scientific information. When the lactoperoxidase-system lost his activation, it's the N_2-flushing that holds the log forming units lower. Growth is possible after 24 hours at 15°C and after 12 hours at 25°C, but N_2-flushing keeps the growth lower, what's positive.

So the conditions with both N_2-flushing and adding one or both substrates thiocyanate and H_2O_2, show lowest results in log forming units of the total aerobic colony count, *Lactobacillus* spp. and *Streptococcus* spp. Of these conditions, condition HTN, with both substrates thiocyanate and H_2O_2 added and N_2-flushing is the best condition to hold the raw milk microflora lower at 15°C and 25°C. Condition HT also shows very low results in counts of each species. So even this condition is very effective.

6 Bioethical reflection

6.1 Economical pillar

By examining the microflora and the growth of these microflora present in raw milk, kept at 15°C-25°C, it's possible to give a positive influence to the economy. By doing research on different methods to keep the initial microflora low in raw milk, the dairy economy can be supported in developing countries. The lactoperoxidase-system and N_2-flushing are examined in the lab to find a good and correct method to keep the initial microflora low between the place of milking on the farm and the factory. In developing countries, milk production methods, equipment and milk-storage aren't developed in many cases. Refrigeration after milking and during transport is not possible in different countries, whereby the initial microflora can grow and the milk quality decreases a lot before further treatments in the factory. In African countries for example, transport of the milk happens by motorbike or by foot. This asks sometimes lots of hours, and at hot temperatures. This isn't useful at all. By examining these methods at the lab, to hold the microflora low by adding some substrates at 15-25°C, less raw milk will be thrown away in these countries because of decreasing qualities. There will be less losses of raw milk that can't be used at the factory trough bad quality and increasing microflora's. In this way the economy will be boost in developing countries.

6.2 Ecological pillar

When looking to the ecological part of this subject, just positive influences will be shown on the ecology in developing countries. By developing new methods to keep the initial microflora's low in raw milk at moderate temperatures of 15°C-25°C, there will be less losses, whereby less raw milk should be thrown away, what's positive for the environment. Otherwise the loads of the nature will increase a lot when liters of raw milk are dumped or thrown away. In this way, more raw milk will reach the factory with a good quality, and more raw milk can be used. When more raw milk can be used at the factory, more industrialized processes will be going on, what's negative on the one side because of the expels of unhealthy substances, etc. But this doesn't compensate the positive influences of this subject. Creating these methods, is for helping the ecological part. Farmers are doing their very best to obtain raw milk with the best quality from their cows, in balance with the nature. Then it's not the intent to throw away lots of raw milk because of the pour circumstances. Important to say, when adding the chemical substrates thiocyanate and H_2O_2 to the raw milk, this hasn't any negative influence on the quality of the milk, or on the environment or the health of the cows. This is already examined. When N_2-flushing is used, there are not yet any proves of the good or bad influences on the environment. So this has to be examined. But it can be said that the subject of this thesis could have a lot of positive influences on the ecological pillar in the developing countries.

6.3 Social pillar

Looking to the social pillar of this thesis is more difficult. One thing can be said, that's the fact of the good treating of the professor and mentor at the university of Helsinki. At the university, the work atmosphere is something completely different. Everyone works more precisely and with more respect for their work. There is a lot of respect between professors and students, they are equal. My mentor helped a lot, so on the social part, there can be made just positive statements. Lots of information and experiences where learned by good communication with the professor and the mentor. The policy of the university is to offer the best to the students and associates.

When looking to the social pillar of the subject of this thesis, it's difficult to make a right conclusion. The methods of the lactoperoxidase-system and N_2-flushing are examined to help the people of the dairy sector in developing countries, so developed countries are searching methods to help the local producers of raw milk at developing countries, what means there is a lot of respect for these people. So doing this research and making conclusions, can help these people for example in African countries. This is just positive. But before there can be a positive influence for these producers, farmers and factories, these methods should be completed and introduced in these countries.

7 Publishable article

Pioneering research in Finland to help the dairy industry in developing countries

In this work, innovative methods to help the dairy industry, especially the producers of raw milk in developing countries, are tested. This research can be interesting for any country, so consumers and producers everywhere in the world have the opportunity to discover these new revolutions! It's not only helping developing countries, it's bringing pioneering information for everybody. These days, the food industry wins more and more attention of the actuality and consumers want to know everything about the origin and composition of food products. Food information becomes more important. But there are also more interests in the way of helping developing countries, where life and facilities are less comfortable. In this research, a perfect combination is made of both topics. This research gives the chance to learn more about the possible methods for giving raw milk a better quality, not just for helping developing countries, but for giving an added value to the food industry worldwide.

Intent

The intent of this research is helping the dairy industry in developing countries. In this work, two different scientific methods are examined to hold the initial microbiota low. In developing countries, there are also a lot of farmers with cows for the production of milk. When the consumer buys milk in the shop, this milk is treated with different heating methods to hold the microbiota and contamination in raw milk low. In this way, the milk can be stored for longer time. When the milk comes straight from the cow, the milk did not have any heating treatment, so there is possibility that the microbiota grows in the untreated milk. To hold this growth low, the milk is normally stored at refrigerated temperatures until it reaches the factory, where heating methods are applied. In developed countries, there are enough facilities to refrigerate the milk until further heating treatments. Transport happens by cooled lorries. In developing countries, the facilities to cool the milk are less developed, in many cases there are just no possibilities to refrigerate the milk. So growth of the microbiota present at the beginning, is possible. To repair this problem, two methods are examined. The first method goes about the effectivity of the lactoperoxidase-system. The second method is N_2-flusing in the headspace of milk flasks.

First method

The first method examined to hold the initial microbiota low, is the lactoperoxidase-system. Lactoperoxidase is an enzyme, present in raw milk. This substance needs to be activated to produce another substance with antimicrobial working. This means that the produced substance inhibits or kills different microbiota's present in the milk. Before this substance can be formed, lactoperoxidase should be activated. In this way, there are two chemical substrates that can be added to activate the lactoperoxidase. These two substrates are called thiocyanate and H_2O_2 (hydrogen peroxide). In this research, experiments are done on the milk by adding the substrates. Or one or both substrates are added to the milk, to control the effectiveness of the substrates to activate the lactoperoxidase. Experiments are done at 15°C and 25°C, because these are the temperatures where the milk is kept in developing countries without possibilities to refrigerate the raw milk after milking and before further heating treatments. When the substrates activate the lactoperoxidase, an antimicrobial substance is formed and holds the initial microflora low, what's really positive for the milk quality. Research is also done on the length of activation of the lactoperoxidase at these different temperatures. In this way, research is done if there is less or longer production of

the antimicrobial component, and there are more possibilities to hold the raw milk longer at temperatures of 15°C and 25°C.

After doing the experiments, it seemed that best results were obtained when both substrates thiocyanate and H_2O_2 (hydrogen peroxide) were added. At 15°C, there was activation of the lactoperoxidase for 24-26 hours, at 25°C, there was activation for 11-12 hours.

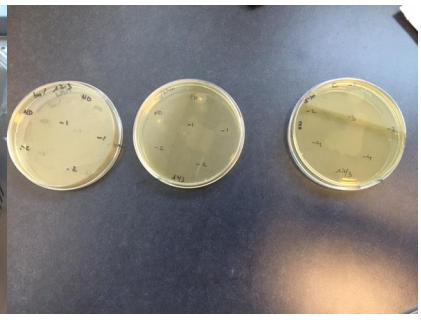
Second method

Another system, examined during this research is N_2-flushing in the headspace of the milk flasks. N_2 is nitrogen gas. During this research, experiments were done with N_2 flushed trough the headspace of the milk flasks. This nitrogen gas also helps to inhibit the growth of different microbiota, but it has still another advantage. There are different products produced in the milk when holding at refrigerated or not refrigerated temperatures. These products are called the lipases and proteases. They cause a bad quality of the milk with disadvantages for the sensory properties and acidifying of the milk (Patricia Munsch-Alatossava, 2009). When N_2-flushing happens, these disadvantages are gone and there is an inhibiting of the growth of the microbiota present in the milk at the beginning.

Experiments were done at 15°C and 25°C. There were also combinations made with the first experiment by adding one or both substrates thiocyanate and H_2O_2 (hydrogen peroxide). Best results were obtained when both substrates, thiocyanate and H_2O_2, are added and N_2-flushing occurs in the headspace of the milk flasks. So combining both methods results in lowest growth of the initial microflora and best sensoric properties following scientific information and research (Patricia Munsch-Alatossava, 2009) . This can really help, both developing and developed countries.

Lab work

The effectiveness of the two methods was examined on three different species of microbiota: the total aerobic colony count, *Lactobacillus* spp. and *Streptococcus* spp. But experiments can be done on more species. The experiments done at the lab were controlled very frequently, in this way correct conclusions could be made. The milk flasks were agitated and hold at 15°C or 25°C in a warm water bath. This is a machine where the temperature of the water is monitored permanently. The initial microbiota was count each time at the beginning. At different moments, the growth of the initial microbiota was controlled by taking a sample of each bottle, and putting this on special made plates to control the growth. These plates are called 'growth media'. The plates were put under specific conditions and temperatures, and in this way it was possible to control the length of the effectiveness of the two methods lactoperoxidase-system and N_2-flushing. A figure of the warm water bath is shown on the left and a figure of a growth media is shown on the right.



After counting the different growths of the samples, graphics and conclusions were made. So people in the dairy industry can consult these results to improve their own products. In this way, developed countries or scientific information can help developing countries with the introduction of these methods. In this way, further steps are taken and more information can

be given. There is no doubt of the good influences this research can have on the dairy industry worldwide.

Conclusion

So both methods examined in this pioneering research, could really help the dairy industry in developing countries where refrigeration after milking is not possible. But also in developed countries it could help the dairy industry to reveal more methods to avoid the disadvantages of some substances in the raw milk.

8 Presentation of the daily work

Monday the 16th of March 2015

- A first acquaintance was made with professor Tapani Allatossava and Patricia Munsch. Appointments were made about the theoretical and practical work of this thesis. More information was given about the experiments and the plan of the 30 days that are coming.
- A guided tour was given in the university and in the lab, where I have to work. Introducal work was done at the lab, to start next day with the experiments.

Tuesday the 17th of March 2015

- The first test was explained by Patricia Munsch. She showed me where I can find and learn more information about lactoperoxidase, to understand the following experiments. More information was given about the first experiment: a lactoperoxidase-test at 25°C.
- The substrates needed for this experiment were made: thiocyanate and H_2O_2.
- More information was given about the different growth-media: MRS, M17 and PCA. The reason why they are used in these experiments.
- The method to make plates was explained, because it happens on a specific way.
- The lactoperoxidase-test at 25°C was set up. To = 9h30-17/3/2015. Plates of the CONTROL-bottle were made on PCA, MRS and M17. There are three conditions: CONTROL, HT and H.
- A first point was made 2h30 hours after To. This is T1 (12h00-17/3/2015). Of each condition, plates of PCA, MRS and M17 were made.
- A second point was made at 18h30. This is T2. Of each condition, plates of PCA and MRS were made.

Wednesday the 18th of March 2015

- Plates of PCA are count for day 1 of points To, T1 and T2 made on 17/3/2015.
- A new counting point is made 24 hours after To (17/3/2015). At 9h30 plates of CONTROL, HT and H are made on PCA, MRS and M17.

Thursday the 19th of March 2015

- Plates of PCA are count for day 2 of points To, T1 and T2 made on 17/3/2015.
- Plates of PCA are count for day 1 of the point 24 hours after To (17/3/2015).
- Following dilutions were made of condition HT 24 hours after To: 10^{-3}, 10^{-4} and 10^{-5}. Because of the uncountable quantities on the PCA-plate of yesterday.
- A last counting point was made 48 hours after To (17/3/2015) of the lactoperoxidase-test at 25°C. Dilutions 10^{-4}, 10^{-5} and 10^{-6} of each condition were put on PCA.
- A new experiment is set up. A lactoperoxidase-test at 15°C with conditions CONTROL, HT, H and T was set up at To = 10h30 – 19/3/2015.

Friday the 20th of March 2015

- Plates of PCA are count for day 3 of points To, T1 and T2 made on 17/3/2015.
- Plates of PCA are count for day 2 of the point 24 hours after To (17/3/2015).
- The plate of condition HT (made on 19/3/2015) is count for the first day.
- Plates of MRS are count for each condition after 3 days of incubation. Plates were made of To, T1 and T2.

- Plates of M17 are count for condition CONTROL after 3 days of incubation. Plates were made of To and T1.
- Plates of each condition were count for day 1 of the point made 48 hours after To (17/3/2015).
- Plates of PCA are count for day 1 of points To and T1 made on 19/3/2015. This goes about the new experiment at 15°C.
- A new counting point was made for the experiment at 15°C, 24 hours after To (10h30-19/3/2015). Plates of PCA were made for each condition.
- New growth media of PCA, MRS and M17 were prepared and sterile plates were made for coming experiments.

Monday the 23th of March 2015

- Results of the experiment at 15°C are noted down after 3 days in the part results. PCA-plates were count of To (10h30-19/3/2015), T1(18h30-19/3/2015), 24 hours after To (10h30-20/3/2015) and 48 hours after To(21/3/2015).´
- MRS-plates of the same experiment were controlled and colonies were count of To(10h30-19/3/2015) and 48 hours after To(21/3/2015). There are no counts of day 3 of this last point.
- M17-plates of the same experiment were controlled and colonies were count of the point 48 hours after To(21/3/2015). There are no counts of day 3 of this last point.
- Final results of the experiment at 25°C were counted and noted down in the part results. For the PCA-plates there were following counting points: To (17/3/2015), T1 (17/3/2015), T2 (18h30-17/3/2015), 24 hours after To(18/3/2015) and 48 hours after To (19/3/2015). For the MRS-plates there were following counting points: To, T1, T2 and the point 24 hours after To. For the M17-plates there were following counting points: To, T1 and the point 24 hours after To.
- A new experiment was set up: a first lactoperoxidase-test at 15°C with N_2-flushing. There are eight conditions in this new experiment: CONTROL, HT, H, T, CN, HTN, HN and TN. To is 9h15- 23/3/2015.
- At To (9h15-23/3/2015) plates of PCA, MRS and M17 are made of the CONTROL-bottle to know the inital microflora´s.
- 2h30 after To a second counting point is made. This is T1 (11h45-23/3/2015). For all conditions, plates of PCA, MRS and M17 are made.
- A following counting point of the first lactoperoxidase-test at 15°C with N_2-flushing was made on PCA and MRS. This is T2 (18h30-23/3/2015).

Tuesday the 24th of March 2015

- The PCA-plates were controlled for the first day of incubation at 30°C of To (9h15-23/3/2015), T1 (11h45-23/3/2015) and T2 (18h30-23/3/2015).
- A new counting point is made 24 hours after To. This is at 9h15-24/3/2015. Plates are made of all conditions.

Wednesday the 25th of March 2015

- Results of the PCA-plates made on To, T1 and T2 were count for the second day. This 3 counting points were made on 23/3/2015.
- Results of the PCA-plates made 24 hours after To (23/3/2015) were count for the first day.
- New plates of PCA, MRS and M17 were made of all conditions 48 hours after To. This is at 9h15-25/3/2015.
- A new experiment is set up with the raw milk of 23/3/2015. There are following conditions: CONTROL, HT (with the old H_2O_2) and HTnew (with the new prepared H_2O_2). This experiment is set up to detect differences in effectivity between the old

H_2O_2 and the new made H_2O_2 on the lactoperoxidase-system. A PCA-plate of the CONTROL-bottle is made at To (10h30-25/3/2015).
- A new counting point of the new experiment was made 2 hours after To (12h30-25/3/2015). This is T1.

Thursday the 26th of March 2015

- New media of PCA, MRS and M17 were prepared for following experiments.
- The PCA-plates (of the new experiment) of To (10h30-25/3/2015) and T1 (12h30-25/3/2015) were count for day 1. These are the counts after one day of incubation at 30°C.
- PCA-plates were count of the first lactoperoxidase-test at 15°C with N_2-flushing. Plates of To, T1 and T2 were count for day 3. These plates were al made on 23/3/2015. The plates made 24 hours after To, were count for day 2. The plates made 48 hours after To were count for day 1.
- The MRS- and M17-plates of To and T2 (made on 23/3/2015) were count after 3 days of incubation at 30°C.
- Plates were count of the extra dilutions made of the counting point 24 hours after To(23/3/2015) for the first day.

Friday the 27th of March 2015

- The PCA-plates (of the new experiment) of To (10h30-25/3/2015) and T1 (12h30-25/3/2015) were count for day 2. These are the counts after two days of incubation at 30°C.
- PCA-plates were count of the first lactoperoxidase-test at 15°C with N_2-flushing. The plates made 24 hours after To (10h30-24/3/2015), were count for day 3. The plates made 48 hours after To were count for day 2.
- Plates were count of the extra dilutions made of the counting point 24 hours after To(23/3/2015) for the second day.
- Results of the MRS-, and M17-plates made 24 hours after To(23/3/2015) were count after 3 days and results were noted above in the part results.

Monday the 30th of March 2015

- Theoretical study is done for the literature study, the necessary completions are done where necessary.
- More information was sought about *Lactobacillus* spp., *Streptococcus* spp. and their compliant growth-media MRS and M17, to better understand the experiments and the intent of using these growth media.

Tuesday the 31th of March 2015

- More information was sought about the lactoperoxidase-system. Patricia gave more information about the legislation of this system and FAO (Food and Agriculture Organization of the United Nations). In this way, it's more efficient to understand the intent of this system.
- Off these information, more information was written.

Wednesday the 1th of April 2015

- The material used for the different experiments is sought in the lab and named, in that way it was possible to correctly describe which material is needed for the lactoperoxidase-test at 15°C and 25°C, and the test with the new H_2O_2. Following

this, the needed methods were also described and worked out. In consultation with Patricia.
- The results of the first lactoperoxidase-experiment at 15°C counted last week were described for each medium and log-forming units were calculated.

Thursday the 2th of April 2015

- The needed tables were made of the results , calculations were made of each condition, each growth media and the different counting points, and comparisons were made. The needed graphics were made and described and showed to Patricia.
- The results of the lactoperoxidase-tests at 15°C, 25°C and with the new H_2O_2 were described and discussed. In that way, partial conclusions can be made.

Wednesday the 8th of April 2015

- A second experiment with N_2-flushing at 15°C is set up. There are eight conditions: CONTROL, HT, H, T, CN, HTN, HN and TN.
  9h40 is To of the experiment. At this hour, plates are made of the CONTROL-bottle on PCA, MRS and M17.
  Three times ND and 10^{-1}-dilution is putted on each plate. The reason for the choice of these dilutions is the freshness of the raw milk. The raw milk comes straight of the university farm.
- A second point is made after 2 hours at 11h40. This is T1. There are plates made of PCA, MRS and M17 for the eight conditions.
  Three times ND and 10^{-1}-dilution is putted on each plate.
- A third point is made at 18h30. This is T2. Of each condition, plates are made on PCA and MRS.
  Three times ND and 10^{-1}-dilution is putted on each plate.

Thursday the 9th of April 2015

- Following media were made today: PCA, MRS and M17. After that, they were autoclaved. And after cooling in the warm water bath, they were put on sterile plates.
- A new point was made of the test with N_2-flushing at 15°C, 24 hours after To (8/4/2015). Plates of PCA were made for each condition.
- Plates were controlled and colony forming units were count of the plates made at To, T1 and T2 on 8/4/2015.
- New tubes were prepared with saline solution, to put in the autoclave. So they are ready to use for following experiments.

Friday the 10th of April 2015

- Plates were controlled and colony forming units were count of the plates made at To, T1 and T2 on 8/4/2015. This is the second day of counting and controlling the plates made at 8/4/2015. In this way, the changing of colony forming units on the plates is controlled each day.
- The plates of PCA, made 24 hours after To, are controlled and colony forming units were count and noted. This is the first day of controlling these plates.
- A point 48 hours after To (8/4/2015) is made. Of each condition plates are made on PCA, MRS and M17.
  Following dilutions are put on each plate for these conditions: three times 10^{-1} and three times 10^{-2} for C, HT, H and T.
  Following dilutions are put on each plate for these conditions: three times ND and three times 10^{-1} for CN, HTN, HN and TN.

- The plates made yesterday with PCA, MRS and M17, are coagulated, putted in sacks and in the refrigeration for later use.

Monday the 20th of April 2015

- A third experiment with N_2-flushing at 15°C is set up. There are eight conditions: CONTROL,HT,H,T,CN,HTN,HN and TN.
  9h00 is To of this experiment. At this hour, plates are made of the CONTROL-bottle on PCA, MRS and M17.
  Three times ND and 10^{-1}-dilution is putted on each plate.
- A first point is made 2h30 after To. This is T1. Of all eight conditions plates are made on PCA.
  Three times ND and 10^{-1}-dilution is putted on each plate.
- The results of the second experiment of the plates with N_2-flushing at 15°C were controlled and noted. The plates were count of the eight conditions on PCA,MRS and M17 at different counting points.
- A second point is made at 18h30. This is T2. Plates are made of each condition on PCA.
  Three times ND and 10^{-1}-dilution is putted on each plate.
- Interpretation and discussion is made and noted of the results and changes of the first test at 15°C with N_2-flushing.

Tuesday the 21th of April 2015

- A counting point 24 hours after To (9h00-20/4/2015) is made of each condition. So the counting point after 24 hours is: 9h00-21/4/2015.
  Of the eight conditions, plates are made on PCA, MRS and M17. For each condition different dilutions are made.
- The results of the colony forming units were controlled of the plates of the third experiment at 15°C with N_2-flushing, at To, T1 and T2. These plates were made the 20^{th} of April. So it´s the first day of counting plates. The results are noted in this document.
- Results are noted and calculations are made of the second test at 15°C with N_2-flushing. Explanation is done of the results.

Wednesday the 22th of April 2015

- Plates were controlled and colony forming units were count of To, T1, T2 and the counting point 24 hours after To. The total aerobic colony count was noted for day 2 for To, T1 and T2. For the point 24 hours after To, it was the first day for counting plates.
  The results are noted in this document.
- Following media were made today: PCA, MRS and M17. After that, they were autoclaved.
- A following counting point is made of the test with N_2-flushing at 15°C, 48 hours after To (20/4/2015). Of each condition plates are made on PCA, MRS and M17. There are put different dilutions on the plates for each condition, depending on the results of the plates made at To, T1, T2 and 24 hours after To.
- More theoretical study is done on the principle and working of N_2-flushing to raw milk. For this, scientific literature is used.

Thursday the 23th of April 2015

- A last point was made of the eight conditions of the experiment at 15°C with N_2-flushing , 72 hours after To. Of each condition, plates were made on PCA, MRS and M17.
- The plates of To, T1 and T2 were count for the last day (day 3). The results were noted and the log forming units were calculated. More explanation was written in this thesis. (of the third experiment at 15°C with N_2-flushing)
- Plates of the points 24 hours and 48 hours after To, were count and noted. (of the third experiment at 15°C with N_2-flushing)
- An extra plate was made of condition H, 48 hours after To with dilutions 10^{-4} and 10^{-5} on PCA. This because of the uncountable growths at dilutions 10^{-1}, 10^{-2} and 10^{-3} yesterday.
- More theoretical study is done on the materials and methods used for the experiment at 15°C with N_2-flushing.

Friday the 24th of April 2015

- Results were count of the plates made, 24 hours after To (20/4/2015). This is day 3 of incubation at 30°C, so the log forming units were calculated of the counts on PCA, MRS and M17. More information is given about the results and noted down in the part results.
- Colonies were count for the second day of the plates made 48 hours after To (20/4/2015). Only plates of PCA were count and results were noted down.
- Colonies were count for the first day of the plates made 72 hours after To (20/4/2015). Only plates of PCA were count and results were noted down.
- The first part of the thesis is discussed and reviewed together with Patricia. Patricia told me about my evaluation.
- More theoretical research is done about the university of Helsinki.

Monday the 27th of April 2015

- A new experiment was set up: a lactoperoxidase-test at 25°C with N_2-flushing. There are eight conditions: CONTROL, HT, H, T, CN, HTN, HN and TN.
  To = 8h00- 27/4/2015
  At To, plates are made of PCA, MRS and M17 of the CONTROL-bottle.
- A first point T1 is made 3 hours after To: 11h00-27/4/2015. Of all conditions, plates are made on PCA.
- A second point T2 is made 7 hours after To: 15h00-27/4/2015. Of all conditions, plates are made on PCA.
- A third point T3 is made 10 hours after To: 18h00-27/4/2015. Of all conditions, plates are made on PCA, MRS and M17.
- The plates made last week at 48 hours and 72 hours after To (20/4/2015), were count of day 3. This goes about the lactoperoxidase-test at 15°C with N_2-flushing. Of these results, the log forming units were calculated and noted down in the part results above. The results of this test were described.
- The discussion is made about the second lactoperoxidase-test at 15°C with N_2-flushing.

Tuesday the 28th of April 2015

- A point is made 24 hours after To (27/4/2015) of the lactoperoxidase-test at 25°C with N_2-flushing. Of each condition, plates are made of PCA, MRS and M17.
- Plates are count for the first day of point To, T1, T2 and T3 of the lactoperoxidase-test at 25°C. Results are noted down above.
- The discussion is made about the second lactoperoxidase-test at 15°C with N_2-flushing.

Wednesday the 29th of April 2015

- Extra dilutions are made of conditions CONTROL, T, H, HT, N, NT and NH yesterday and extra plates of PCA are made of these conditions 24 hours after To. This because of the uncountable quantities of the total aerobic colony counts of these dilutions put on the plates yesterday (8h00-28/4/2015).
- Colonies are count for day 2 of the plates made at To, T1, T2 and T3 and results are noted down above in the part results.
- Colonies are count for day 1 of the plates made 24 hours after To. Results are noted down above in the part results.
- New growth media were prepared of MRS and M17 and sterile plates were made.
- Preparatory work is done for the next experiment that has to come.
- The discussion is made about the third lactoperoxidase-test at 15°C with N_2-flushing.

Thursday the 30th op April 20105

- New sterile material was prepared for the following experiment.
- A first version of this thesis was overlooked with Patricia, she gave me her evaluation and comments.
- The results were controlled and noted down for day 3 of the plates of PCA, MRS and M17 of counting points To, T1, T2 and T3 on 27/4/2015. The log forming units were calculated and the results were described above.
- The results were controlled and noted down for day 2 of the PCA-plates made 24 hours after To (27/4/2015).
- The discussion is made about the third lactoperoxidase-test at 15°C with N_2-flushing.

Monday the 4th of May 2015

- Results of day 3 of the PCA-plates made 24 hours after To (27/4/2015), are counted and noted down in the part results. This goes about the first lactoperoxidase-experiment at 25°C with N_2-flushing. Log forming units were calculated. The results were also described.
- Results of day 3 of the MRS- and M17-plates made 24 hours after To (27/4/2015) are counted and noted down in the part results. Log forming units were calculated. All results of the different counting points of these plates were described.
- Graphics of the three media were made and described of the results that show differences between the eight conditions in the first lactoperoxidase-experiment at 25°C with N_2-flushing.
- A PCA-plate was made of the fresh raw milk that's gonna be used in the second lactoperoxidase-test at 25°C with N_2-flushing. The second experiment will be lanced in the evening so this plate is made to remark differences in the colony counts between this point made at 9h00-4/5/2015 and To in the evening on 4/5/2015.
- The discussion is made about the first lactoperoxidase-experiment at 25°C with N_2-flushing.
- A second lactoperoxidase-test is set up. To= 20h30-4/5/2015. A PCA-, MRS-, and M17-plate are made of To.

Tuesday the 5th of May 2015

- The PCA-plate made yesterday morning of the raw milk used for the second lactoperoxidase-test at 25°C, is controlled and results are noted down. This is NOT To, this plate is just for controlling the differences between the results at this point and at To.

- The PCA-plate of To was count for day 1 but no colony counts were detected. These results are noted above in the table.
- A new counting point was made after 12 hours (8h30-05/05/2015). This is T1. Plates of all conditions were made on PCA, MRS and M17.
- T2 was made 17 hours after To (04/05/2015). T2= 15h30-05/05/2015. Plates of all conditions were made on PCA, MRS and M17.
- Plates of PCA, MRS and M17 were made of all conditions 24 hours after To. 20h30-05/05/2015) = 24 hours after To.

Wednesday the 6th of May 2015

- The PCA-plate made Monday morning (09h00-04/05/2015) of the raw milk used for the second lactoperoxidase-test at 25°C, is controlled for day 2 and results are noted down.
- The PCA-plate of To (20h30-04/05/2015) was count for day 2. The results are noted above in the table.
- The PCA-plates made on T1 (8h30-05/05/2015) and T2 (15h30-05/05/2015) of all conditions, were controlled and counted for day 1. Results are noted in the table above.
- The PCA-plates made 24 hours after To (=20h30-05/05/2015) of all conditions, were controlled and counted for day 1. Results are noted in the table above.
- There is thought about a correct explanation of the intention of this thesis.

Thursday the 7th of May 2015

- The PCA-plate made Monday morning (09h00-04/05/2015) of the raw milk used for the second lactoperoxidase-test at 25°C, is controlled for day 3 and results are noted down. The log forming units at this point are calculated and the results are described above.
- The PCA-plate of To (20h30-04/05/2015) was count for day 3. The results are noted above in the table. The initial microflora of the total aerobic colony count was calculated and the results were described.
- The MRS-plate and the M17-plate made at To (20h30-04/05/2015) are counted after 3 days of incubation. Log forming units of *Lactobacillus* spp. and *Streptococcus* spp. are calculated and the initial microflora´s of both species are described above.
- The PCA-plates made on T1 (8h30-05/05/2015) and T2 (15h30-05/05/2015) of all conditions, were controlled and counted for day 2. Results are noted in the table above.
- The PCA-plates made 24 hours after To (=20h30-05/05/2015) of all conditions, were controlled and counted for day 2. Results are noted in the table above.
- More explanation and questions were asked to Patricia, to better understand the research of this thesis.

Friday the 8th of May 2015

- The PCA-plates made at T1 (8h30-05/05/2015) and T2 (15h30-05/05/2015) of all conditions, were controlled and counted for day 3. Results are noted in the table above. Log forming units are calculated and the results are described above.
- The PCA-plates made 24 hours after To (=20h30-05/05/2015) of all conditions, were controlled and counted for day 3. Results are noted in the table above. Log forming units are calculated and the results are described above.
- The MRS-plates and M17-plates made at T1, T2 and 24 hours after To, are controlled and counted. The results are written above. Log forming units are calculated for both media and results are described.

- Graphics of the 3 media are made, where a comparison can be made between the different conditions at different counting points.
- The discussion is done about the second lactoperoxidase-test at 25°C with N_2-flushing.

9 Personal opinion

9.1 Personal opinion about the university

During this internship at the university of Helsinki, I learned a lot about the differences in working and strategies between Finland and Belgium. The way of working is more enhanced and controlled in Finland. The lifestyle of the people and students working at the university of Helsinki is more restful whereby they work more detailed following me. In the microbiology lab of the dairy group, there was a good communicating between people working in the lab. In Belgium, people are more stressed at work, they hurry all the time. Here at the university, professors and lectors are working more concerned. When something doesn't work like it should be, there are no problems and the day after, everything will be repeat in the same way. In contrast with the Belgian mentality, where everything has to go fast, even when it's not finished in the way it should be.

So here at the university of Helsinki, in my vision, the way of working is much better. They work more detailed, they show more respect for students and associates and they try to offer the best possibilities for everyone. There is also more respect for the environment, by strictly sorting every kind of material and trashes. They try to use as less as possible polluting material such as plastic. So they are working very economical, with respect for each member of the university and the environmental part.

The university of Helsinki tries to be part of the top 50 best universities of the world, by offering the best educational programs and publishing the best scientific literature information. That's one of their biggest visions and I think they are doing good in this way. I just saw one part of the university, but if each part of the university works in this way, their goal is possible. This university was a very nice place to have a two months during internship, everyone tried to offer the best possibilities to their own and foreign students.

9.2 Personal opinion on the internship

This internship was an enriching challenge, which gave me just positive experiences. First of all, it was very interesting to do this work in a foreign language, that gave me more possibilities to enhance my French and English. I talked French with my mentor Patricia Munsh-Alatossava and I wrote my thesis in English, so I'm now more experienced in talking and writing scientific information in other languages, which will support me in my future work opportunities. My mentor corrected when needed, so it was possible to make the very best of this internship.

Before leaving for Finland, I didn't know really good what the subject would be at the university of Helsinki. I knew I had to work at the dairy lab, what already was very interesting. But I didn't know much about the subject of the lactoperoxidase-system and N_2-flushing. It was a very interesting subject because it was something brand new. I never heard about it before, so in that way I just learned new things. And that was also the intent of my internship, expanding my knowledge. Doing the experiments at the lab gave me some responsibility because results were depending on me, so I tried to be as correct as possible. The way of working at the university of Helsinki was more detailed, whereby my way of working also changed a lot. What's just positive.

Important to say is that the working part at the lab and the completing of my thesis, was regarded as a master thesis work in my opinion. The level was something completely else. There was more communication between me and the mentor, but at the same time, I had to do much more on my own way, making conclusions by myself, writing and giving statements

about this to my mentor, etc. I was glad to work in this way because I had the feeling to give an added value. So working in this sector, would be very interesting, doing experiments, making comparisons and giving new information to the sciences. It's something challenging.

This internship gave me the possibility to learn more about foreign cultures and working methods, but also to enhance my knowledge and skills in each way of working. It was a challenge for me to write this thesis in English, but it gave me just positive feelings. The dairy sector is something where still new things can be explored, it makes me curious to still learn more about it.

Just one conclusion can be made, this internship at the university of Helsinki was a very interesting and exploring work, where I learned a lot of things in many ways.

Appendix

List with abbreviations

Specification of the abbreviations:

CONTROL:
Just raw milk is added to the test-bottle -no chemical products are added to the raw milk.
HT:
Both substrates H_2O_2 and thiocyanate are added to the raw milk.
H:
Only substrate H_2O_2 is added to the raw milk.
T:
Only substrate thiocyanate is added to the raw milk.
CN:
N_2-flushing is added to the raw milk in the test bottle.
HTN:
Both substrates H_2O_2 and thiocyanate are added and also N_2-flushing is added to the raw milk in the test bottle.
HN:
Substrate H_2O_2 and N_2-flushing are added to the raw milk.
TN:
Substrate thiocyanate and N_2-flushing are added to the raw milk.
ND:
Not diluted.
NC:
No colony forming units detected on the plate.
≥ :
There are too much colony counts to detect precisely.
PCA:
Plate Count Agar
MRS:
De Man, Rogosa and Sharpe
N_2:
Nitrogen gas

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