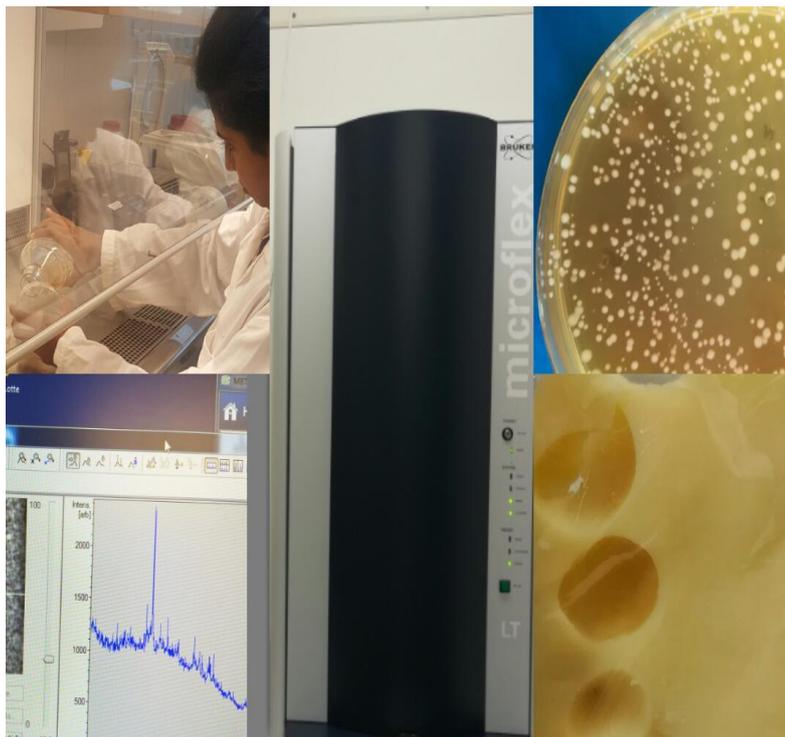


# Identification of microbial composition of Swedish hard cheese by MALDI ToF mass spectrometry

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Department of Molecular Sciences

Independent Project in Food Science • Bachelor Thesis • 15 hec • First cycle G2E

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## Abstract

This study was done with four different Swedish cheese samples in terms of cheese variety, brand and sensorial properties. The objectives of the current study were to identify the different microorganisms present in the Swedish hard cheese by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI ToF MS) and to test the suitability of the method to be use for cheese. And also, to identify differences between good and bad sensorial quality hard cheese in terms of microbial composition. Four different cheese samples were cultivated on four different agar media under both aerobic and anaerobic conditions and the resulted colonies were identified by MALDI ToF MS. This technique allows to identify most of the bacteria only up to species level due to the limitation of database on subspecies and strains levels and some of the microorganisms stayed unidentified. The log colony forming units per gram of cheese was not significantly different among cheese A-good, A-bad, B and C. However, the microbial composition was different for all studied cheese samples. Also, the good and bad sensory quality cheeses were significantly different in terms of microbial composition and the method can be considered as the first tool for deeper investigation of cheese microbiota.

*Keywords:* MALDI ToF MS, microbial identification, cheese, microbial quality, starter culture bacteria



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## Abbreviations

cfu	Colony forming units
HACCP	Hazard analysis and critical control point
LAB	Lactic acid bacteria
MALDI TOF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
PBS	Phosphate-buffered saline
PCA	Plate count agar
PMF	Peptide mass fingerprint
TSA	Tryptic soy agar



# 1 Introduction

Cheese is a popular food item among dairy products. At present, large number of cheese varieties are produced and many scientists are involved in studies and researches on cheese with range of scientific disciplines: milk biochemistry, chemical properties of cheese constituent, enzymes, microbiota, genetics, nutrients, toxic compounds, rheology, flavour and chemical engineering related with cheese production (McSweeney et al., 2004). Microbiota is highly related with cheese quality and safety. According to the hazard analysis and critical control points (HACCP) plans, control of milk microbiota which is used at dairy plants is crucial. Therefore, rapid methods of microbiota identification is a must in cheese industry (Papademas, 2014).

Swedish cheeses are very popular in Swedish dairy sector. *Vasterbotten*, *Pras-tost*, *Greve*, *Herrgardsost*, *Blagotland*, *Svecia* and *Hushallsost* like cheese types are some of them. Current study was conducted by using three types of Swedish long ripened cheese, and they are named hereafter as cheese A, B, C. In cheese A, two cheese types as good and poor were studied, in terms of sensorial properties. Cheese B and C are two commercial brands of another cheese type available in Sweden.

Hence as the cheese texture and quality are highly related with biochemical reactions promoted by starter- and secondary microbiota, identification of microbial composition with rapid, accurate techniques are very important (Beresford et al., 2001). Today, most popular and highly used microbial identification methods are DNA or RNA based molecular methods. However, these methods need trained laboratory personnel to run the process and DNA sequencing methods are expensive and not suitable for routine analyses. Therefore, matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI ToF MS) is suggested as a good way of microbial identification in cheese industry. It gives rapid results within few minutes with a higher accuracy. Very small quantity of microbiota is enough for identification. And, the analyses costs are relatively lower compared with other molecular techniques (Angeletti, 2017).

The main objectives of this study were to identify microbial composition of different types of Swedish hard cheese by MALDI ToF MS and to test the suitability of the method to be used on cheese as a matrix. And, to identify the differences between good and bad sensorial hard cheese in terms of microbial composition.

Hypotheses of the study were:

- Different types of Swedish hard cheeses contain different microorganisms
- Microbial composition is different between good and bad quality hard cheese of same cheese type

## 2 Literature Review

### 2.1 Cheese

Cheese is a highly diverse dairy food item which was initially developed as an attempt to preserve milk. Today, around 1000 varieties of cheeses are produced in different areas of the world (O'Mahony and Fox, 2014). Classification of cheese is not an easy task due to its complexity and diversity. Therefore, most of the cheese classification systems are based on one factor or combination of few factors such as textural characters, source of milk, method of coagulation, ripening agent, temperature used for cooking and composition of cheese (Almena-Aliste and Mietton, 2014).

Based on the moisture content present in the cheese, cheese can be classified into groups as extra hard, hard, semi-hard, semi-soft and soft. Extra hard and hard cheeses are ripened for a longer period of time and therefore, contains very less moisture content.

In some of the cheese varieties, starter culture bacteria accumulate in the macroscopic cavities of grains in which whey contains, when the grains are collected below the surface of the whey. Initially the produced gas dissolve in liquid and with continuous bacterial growth, local super saturation occurs and small holes are created. These holes are called for eyes.

### 2.2 Factors influencing the quality of the cheese

Cheese quality is a broad term and it is mainly related with the appearance, texture, flavour, functionality and nutritional value. Quality of the final cheese is determined by numerus production steps and manufacturing process with different conditions.

Initial microorganisms present in the milk and secondary microorganisms affect on the cheese quality. And, ripening temperature and length, the quality of raw milk also affect on final quality of the cheese (Fox et al., 2017). Microbiota presents in the cheese determine the future of cheese characters, shelf-life and safety of the product (Irlinger and Mounier, 2009).

### 2.3 Methods to identify microbiota in milk and cheese

As the cheese quality and shelf life is highly associated with the microbial composition of the cheese, it is important to identify the microbiota present in the final product as well as the raw milk. There are three methods of identification of microorganisms. First method is the cultivation of microbiota followed by phenotypic characterisation and the second is cultivation, followed by molecular characterisation. The third method is dependent on only molecular characterisation without cultivation. Classical cheese microbiology is mainly dependent on the first method. In that, homogenised cheese samples are cultivated on a range of media and then isolated colonies are further characterised. However, this method is unreliable due to the dependency of phenotypic characters, on culture media and environment. Sensitivity is also limited in this method; therefore, this cannot be used to differentiate subspecies or strains. Molecular methods overcome these limitations related to phenotypic characterisation. However, culture dependent molecular characterisation has the risk of assessing only a fraction of the microbial community. Therefore, culture independent molecular methods should be further introduced for cheese microbial identification (Beresford et al., 2001).

### 2.4 Sensory properties of the cheese

Consumer acceptance is the most important factor which should be considered when producing a product to market. Most important sensory properties are taste, aroma and texture. Lipolysis and proteolysis have an impact on the flavour of cheese (Grappin and Beuvier, 1997). Moisture, protein and fat content is the major determinants of cheese texture (Zheng et al., 2016). These aspects can be evaluated by sensorial panels or using instruments. In the food industry pH meters, acidity meters, penetrometers, texture analysers, electronic tongues and noses like instruments are used to evaluate these characters.

## 2.5 Beneficial microorganisms

In generally starter culture can be defined as a specific microorganism or collection of microorganisms which are inoculated directly into food material to obtain the expected fermentation and by that to achieve the desired development of the product. These changes are the improvement in keeping quality, enhanced food safety, improvement in nutritional value, increase in sensory qualities and economical value in the final product (Hutkins, 2008). Most of the time lactic acid bacteria (LAB) are used as starter cultures. The most common LAB among many others are *Lactococcus*, *Lactobacillus*, *Leuconostoc* and Propionic acid bacteria.

*Lactococcus* are gram positive, spherical shaped, facultative anaerobic, non-spore forming, homofermentative bacteria belonging to *Streptococcaceae* family. *Lactococcus lactis* (*L. lactis*) plays a major role in the dairy industry as a probiotic starter culture.

*Lactobacillus* are gram positive, rod shaped bacteria belonging to *Lactobacillaceae* family. They are symbiotic anaerobic bacteria (George and Raizul Haque, 2013). *Lactobacillus paracasei* (*L. paracasei*) is an important species used in dairy industry and can be found in human and animal gut (Smokvina et al., 2013). This LAB act as an probiotic strain and provide health benefits (Desmond et al., 2004)..

*Leuconostoc* are gram positive, facultative anaerobic, heterofermentative, ovoid shaped cocci belonging to *Leuconostocaceae* family. *Leuconostoc* species are important in food and beverage industries due to their capability of improving nutritional and organoleptic qualities and shelf life (Caplice and Fitzgerald, 1999). They produce eyes in cheese due to carbon dioxide production and contribute to the characteristic flavours in cheese due to flavour compounds (Hemme and Foucaud-Scheunemann, 2004). These species produce diacetyl which contributes for the flavour in cheese by citrate metabolism (Starrenburg and Hugenholtz, 1991).

*Lactococcus*, *Lactobacillus* and *Leuconostoc* have the ability to convert lactose into lactic acid and therefore, they are used as starter cultures. *Propionobacterium* and *penicillium* like other microorganisms are also used in cheese making. Hence they do not involve in acid production, they are called as secondary cultures. Their major role is production of organoleptic and biochemical changes in cheese (Fox et al., 2004).

Propionibacteria is gram positive bacteria, which prevents the moulds and gram-negative bacterial growth. Carbon dioxide produced by these bacteria reduces the pH and make an anaerobic environment which is not favourable for aerobic spoilage microorganisms. In most of Swiss cheese productions, propionibacteria is the cause for characteristic “eyes” presence in cheese due to production of carbon dioxide. Size of these eyes are changed by manufacturers by controlling acidity, temperature, and curing time of the mixture (Caplice and Fitzgerald, 1999).

## 2.6 Non beneficial microorganisms

*Pseudomonas* species have the ability to decompose protein and fat by using their enzymes such as proteinases and lipases respectively at the low temperatures. This phenomenon results in bitter flavour in the milk used for cheese production. Coliforms make the early blowing and disagreeable taste which reduces the quality of the final production. These bacteria should be eliminated by sufficient pasteurization process. *Clostridium tyrobutyricum* like bacteria cause serious problems due to their capability of spore formation and survival of spores under pasteurization. They ferment the lactic acid and produce butyric acid which has an unsavoury taste. Also hydrogen gas is developed during the maturation, which destroy the cheese texture (Bylund, 1995).

## 2.7 MALDI ToF method

### 2.7.1 Applications in microbial diagnosis by MALDI ToF method

Current application of MALDI ToF MS is relatively higher in clinical microbiological sector. This method has the ability to identify bacteria up to species level in most of the clinical laboratories in very few minutes. Identification of viruses, identification of mutations, screening of virus subtypes from clinical samples and using them in molecular epidemiology is another application of this method in clinical sector (Cobo, 2013).

Researches based on lipid and phospholipid identification (Schiller et al., 2004), identification of pathogens from patients' blood and urine samples (Wieser et al., 2012), amide hydrogen exchange measurements (Mandell et al., 1998) are most of the aspects currently tested with MALDI ToF MS.

Identification of pathogenic as well as beneficial microorganisms in food and dairy sector by MALDI ToF MS has been increased in the past few years (Giebel et al., 2010). Microbial identification can be done by either comparing the peptide mass fingerprint (PMF) of unknown organism with the PMFs contained in the database or comparing the masses of biomarkers of unknown organism with proteome database (Singhal et al., 2015).

### 2.7.2 Advantages and disadvantages of MALDI ToF method

The main advantage of MALDI ToF method is its rapidity. It gives the results within few minutes. MALDI ToF MS is sensitive. It has the ability to identify the

microorganism from very low amount of material and also it can identify bacteria as well as viruses and fungus (Angeletti, 2017).

A main drawback of MALDI ToF method is limitation in identification due to limited database which makes it difficult to identify some of the microorganisms up to strain level. Therefore, the data base should be always updated with the details of new microbial spectra (Cobo, 2013). This method has a higher initial cost due to the MALDI ToF equipment (Singhal et al., 2015).

## 3 Materials and Methods

### 3.1 Cheese samples

Four types of cheese samples were used for the study. Cheese A-good, A-bad, B and C. Cheese A was a hard, granular eyed cheese which was ripened for one year. A-good and A-bad were two different samples of cheese A, in terms of sensorial properties. Cheese A-good was identified as good sensorial cheese and cheese A-bad was identified as bad sensorial quality cheese by a sensorial panel of a popular Swedish dairy company. Cheese B and C were two different brands of semi-hard, round-eyed cheese of same type, ripened for ten months. Cheese samples were stored in a cold room at 4°C before use.

### 3.2 Phosphate-buffered saline solution preparation

Phosphate-buffered saline (PBS) solution was prepared according to the standard protocol (Cold spring harbor PBS protocol). 500 ml of prepared PBS solution was stored at 27°C in a laboratory glass bottle after autoclaving at 121°C for 30 minutes (CertoCLAV A-4050 autoclave, CertoClav Sterilizer GmbH, Traun, Austria). pH of the PBS solution was 7.2.

### 3.3 Agar media preparation

Four types of agar media were used to obtain as much as possible of the microorganisms present in the cheese. Rogosa agar (pH 5.5; Merck KGaA, Darmstadt, Germany) was used as a *Lactobacillus* selective agar medium and M-17 (pH 6.9; OXOID Ltd., Basingstoke, Hampshire, England) was used as *lactic streptococci* selective medium. Tryptic soy agar (TSA) (pH 7.3) and plate count agar (PCA)

(pH 7.0; Sigma brand products, St. Louis, USA) were used as non-selective media. All agar media were prepared according to the manufacturer's instructions and autoclaved at 121°C for 15 minutes (CertoCLAV A-4050 autoclave, CertoClav Sterilizer GmbH, Traun, Austria). After that, agar solutions were poured into plates under sterilized conditions and stored in 2°C.

### 3.4 Sample preparation

Three gram of cheese sample was cut from the core of each cheese bulk separately and the cheese sample was placed into 50 ml falcon tube. Then 27 ml of PBS solution (pH 7.2) was added to the falcon tube. After the sample was shaken (INFORS HT shakers, Switzerland) at 0.24 g force for 30 minutes at 37°C. Resulted sample was considered as  $10^0$  concentrated solution. 100 µl from  $10^0$  concentrate was transferred into the Eppendorf safe lock tube (Eppendorf, Germany) with 900 µl PBS solution using a 1000 µl micro pipette to prepare the  $10^{-1}$  diluted solutions. Then the dilutions of  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  and  $10^{-5}$  were also prepared by mixing 100 µl of higher concentrates with 900 µl of PBS solution. After that 100 µl of each solution was pipetted to four plates from each agar medium to grow under anaerobic and aerobic conditions and spread by inoculation spreader (SARSTEDT, Germany) under sterilized conditions. However, all the concentrations;  $10^0$ ,  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  and  $10^{-5}$  were prepared only for cheese A-good and cheese A-bad which were incubated under anaerobic conditions in rogosa and PCA media to identify the best concentration for microbial growth. Therefore, initially 48 plates were prepared from cheese A-good and A-bad. After that,  $10^0$  and  $10^{-1}$  were identified as best concentrations for proper microbial growth by observation. After that, 112 plates of cheese B and C in all four media under anaerobic and aerobic conditions, cheese A-good and A-bad in TSA and M-17 media under anaerobic and aerobic conditions and cheese A-good and A-bad in rogosa and PCA media under aerobic condition were prepared only from  $10^0$  and  $10^{-1}$  concentrations. Altogether, 160 plates were prepared.

### 3.5 Incubation of samples in anaerobic and aerobic condition

The duplicated concentrations of each agar medium were incubated in both aerobic and anaerobic conditions under 37°C for 72 hours. For anaerobic conditions the anaerobic jars with microbiology anaerocult (Merk KGaA, Darmstadt) were used.

### 3.6 Colony forming units and re-cultivation by streaking method

The number of colonies and log colony forming units (cfu)/gram of cheese were calculated only for  $10^{-1}$  concentration, hence it had a considerable microbial growth with a proper density for counting. By considering morphological differences like size and shape, some of the randomly selected colonies from both  $10^0$  and  $10^{-1}$  concentrations were inoculated to new plates by streaking method under sterilized conditions. The colonies from anaerobic and aerobic conditions were again incubated in anaerobic and aerobic conditions respectively.

### 3.7 Microbial identification by MALDI ToF MS

Six colonies from each agar media in each cheese type under anaerobic and aerobic conditions were analysed. Initially, 96 colonies under anaerobic condition for all four cheese types in all four agar media and only 48 colonies under aerobic condition for cheese B and C in all four agar media were analyzed. After that, additional 20 colonies were analyzed for the plates in which the score value for identification was less and in which some organisms were presented in very low frequencies. Altogether, 164 colonies were analyzed. Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI ToF MS) (269944.01850, Bruker Daltonics GmbH., Germany) was used for microbial identification, combined with software (MBT Compass). A re-cultivated colony was slightly touched by a toothpick and it was streaked on a circle of the target. Each colony was run in duplicates and therefore, 328 identifications were done. After that, 1  $\mu$ l of matrix was put on the circle by a micro pipette. After the matrix was dried, it was inserted into the MALDI ToF MS. The results were compiled by the software connected to instrument and database within few minutes.

### 3.8 Statistical analyses

Average of the cfu of  $10^{-1}$  concentration of all the media under both aerobic and anaerobic conditions were calculated for all four cheese types. These averages were considered as replicates and mean log cfu/g of cheese were calculated for all four cheese types under both anaerobic and aerobic conditions (Tukey pairwise comparison, Minitab 17.3.1 version). The principal component analysis was done for the different microbial organisms grown in different cheese types (OriginPro 2017). Percentage of different microbial content of a cheese was calculated based on the identification results given by MALDI ToF MS.

## 4 Results

### 4.1 Number of colonies formed by different cheeses in anaerobic conditions

Mean values of log cfu/g of cheese of A-good, A-bad, B and C cheeses were significantly not different in anaerobic conditions (Table 1).

Table 1. Mean log colony forming units (cfu) per gram of cheese for four different cheeses in  $10^{-1}$  concentration under anaerobic conditions

Cheese type	Mean (log cfu/g of cheese)
A-good	$4.85 \pm 0.40^a$
A-bad	$5.21 \pm 0.10^a$
B	$5.45 \pm 0.76^a$
C	$5.82 \pm 0.40^a$

Values within a column having different superscripts are significantly different ( $p < 0.05$ )

### 4.2 Number of colonies formed by different cheeses in aerobic conditions

The log cfu/g of cheese for cheese A-good and cheese A-bad were not significantly different. Also, no significant difference was observed for cheese C and cheese B (Table 2). However, log cfu/g of cheese in cheese A-good and A-bad were significantly different from cheese B and C ( $p < 0.05$ ). There was no colony growth on the agar plates when homogenate from cheese A-good and cheese A-bad in aerobic conditions were tested.

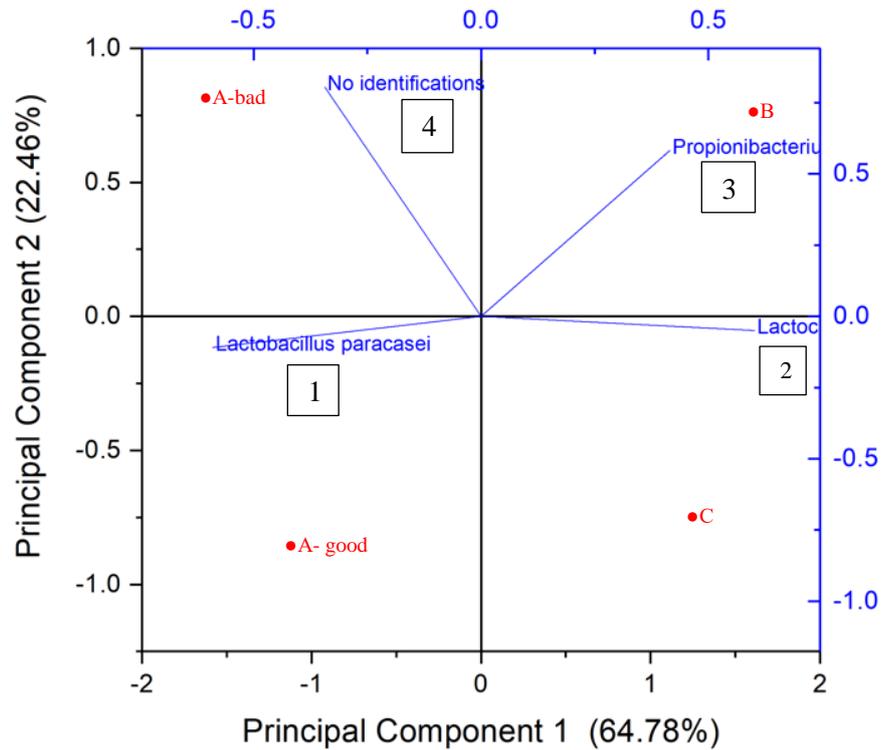
Table 2. Mean log colony forming units (cfu) per gram of cheese for four different cheeses in  $10^{-1}$  concentration under aerobic conditions

Cheese type	Mean (log cfu/g of cheese)
A-good	$0.00 \pm 0.00^a$
A-bad	$0.00 \pm 0.00^a$
B	$4.81 \pm 3.21^b$
C	$5.72 \pm 0.50^b$

Values within a column having different superscripts are significantly different ( $p < 0.05$ )

### 4.3 MALDI ToF MS analyses for colonies in anaerobic conditions

The distribution of microorganisms grown in different cheeses during anaerobic conditions is shown in Figure 1. Highest presence of each microorganism was: *L. paracasei*, in cheese A-good; *L. lactis*, in cheese C and *Propionibacterium freudenreichii* (*P. freudenreichii*), in cheese B. Cheese A-good contained only *L. paracasei*. Cheese A-bad contained 81.25% of *L. paracasei* and 18.75% of unidentified organisms. Cheese B contained 87.50% of *L. lactis*, 4.17% of *L. paracasei*, 4.17% of *P. freudenreichii* and 4.17% of unidentified organisms. Cheese C contained only *L. lactis*.

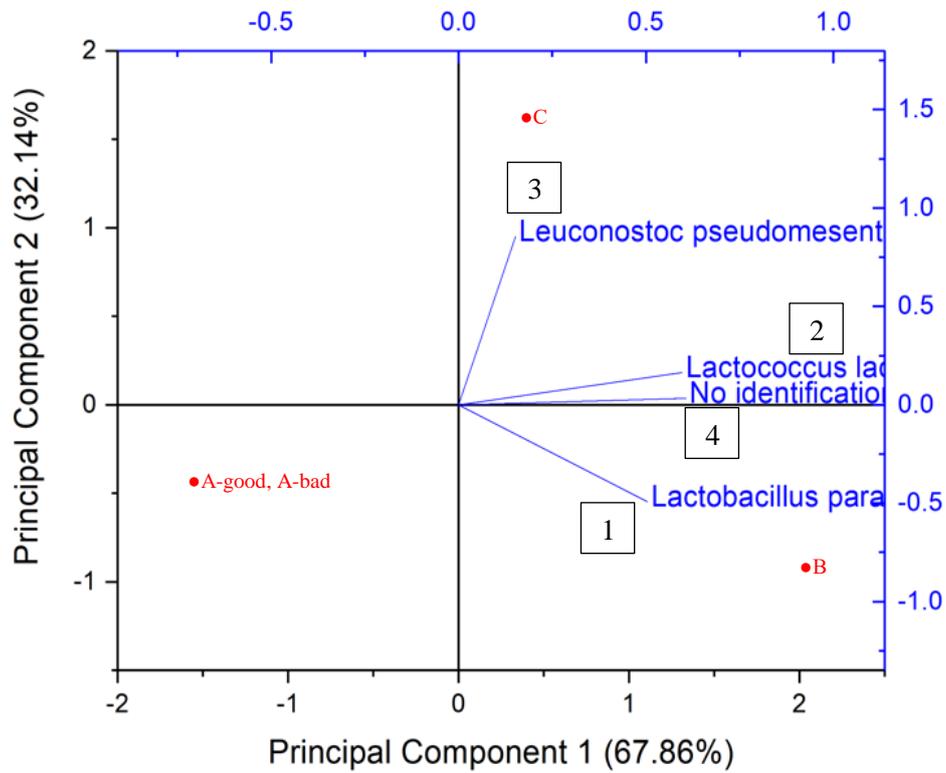


1-*Lactobacillus paracasei*                      2-*Lactococcus lactis*  
 3-*Propionibacterium freudenreichii*      4-No identifications

Figure 1. Principal component analysis for different microorganisms identified by MALDI ToF MS in four cheese types in anaerobic conditions.

#### 4.4 MALDI ToF MS analyses for colonies in aerobic conditions

The distribution of microorganisms grown in different cheeses during aerobic conditions is shown in Figure 2. Highest presence of each microorganism was: *L. lactis* and *L. paracasei* in cheese B and *Leuconostoc pseudomesenteroide* (*L. pseudomesenteroide*) in cheese C. Cheese A-good and A-bad did not have any colony in aerobic conditions. Cheese B contained 79.17% of *L. lactis*, 8.33% of *L. paracasei* and 12.5% of unidentified organisms. Cheese C contained 61.54% of *L. lactis*, 30.77% of *L. pseudomesenteroide* and 7.69% of unidentified organisms.



- 1-*Lactobacillus paracasei*
- 2-*Lactococcus lactis*
- 3-*Leuconostoc pseudomesenteroide*
- 4-No identifications

Figure 2. Principal component analysis for different microorganisms identified by MALDI ToF MS in four cheese types in aerobic conditions.

Microbial growth in different agar media in anaerobic and aerobic conditions were differ from each other and details of different microorganisms presented in rogorosa, M-17, PCA and TSA are shown in Appendix 1.

## 5 Discussion

### 5.1 Colony forming units in different cheeses under anaerobic conditions

Present study suggested that there was not significant difference between A-good, A-bad, B and C cheeses in terms of log cfu/g of cheese incubated in anaerobic conditions ( $p < 0.05$ ). The cheese B and C contained same amount of cfu irrespective of their different brands and other production aspects. Possible reason to observe the same level of cfu/g of cheese in all cheese types could be as explained by Souza et al. (2003). According to the authors microbiota content reaches to their highest presence in the cheese after seven days of maturation and after that it reduces due to lactic acid production followed by reduction of pH. However, the cheese A-good and bad also had the significantly similar amount of log cfu/g of cheese as B and C. As Grappin and Beuvier (1997) have explained, the most important sensory properties as taste, aroma, texture and flavor are affected by lipolysis and proteolysis. According to the Zheng et al. (2016), moisture, protein and fat content determine the cheese texture. According to the Starrenburg and Hugenoltz (1991), *Leuconostoc* species contribute for the flavor in cheese by citrate metabolism. Hutkins (2008), has explained that starter culture bacteria enhance the sensorial properties of cheese. These findings demonstrate that sensorial quality depends on many factors as well as the microbial composition. In other hand, log cfu/g of cheese gives an idea about the total amount of microbiota. This microbiota can be consisted with different species, subspecies or strains. Therefore, log cfu/g of cheese can be same even in sensorial different cheeses.

## 5.2 Colony forming units in different cheeses under aerobic conditions

There was not significant difference between cheese B and cheese C, incubated in aerobic conditions. Although *L. paracasei* presented in both cheese A-good and Cheese A-bad under anaerobic conditions, there were no colony growth in the samples from cheese A-good and A-bad in aerobic conditions. George and Raizul Haque (2013) discussed that *Lactobacillus* species are symbiotic anaerobic bacteria. Also, Cerbo and Palmieri (2013) have described *L. paracasei* as a gram-positive, nonsporing and nonaerobic bacteria and however, they are aero-tolerant. These results prove that aerobic conditions are not best for growth of most of the *Lactobacillus* species.

## 5.3 MALDI ToF MS analyses for colonies in anaerobic conditions

Principal component analysis (Figure 1) gives an overview about microbiota composition in all studied cheese types. It showed that four different cheese types were differed in terms of the microorganisms present in the cheese. Although the log cfu/g of cheese of four different cheese types were not significantly different, the microorganism species presented in the four different cheeses were different. Due to limitation of MALDI ToF MS databases, there were some unidentified microorganisms. The highest present could be seen in cheese A-bad. We speculate that this could be one of the potential reasons for lower sensorial scores for cheese A-bad. Present study is a part of ongoing research project at the dairy research group of Swedish University of Agricultural Sciences (SLU) and the study was performed within a limited time period. Therefore, further identification of unidentified organisms will be continued at next phases of the project. *P. freudenreichii* were presented only in cheese B. This cheese is of type round-eyed cheese. It is in agreement with Caplice and Fitzgerald (1999), showing that *Propionibacterium* species produce carbon dioxide which results the characteristic eye formation of round-eyed cheeses *L. lactis* highly presented in cheese C. *L. lactis* is a major starter culture bacteria used in cheese industry for the lactic acid fermentation (Song et al., 2017). In cheese A-good, all the analyzed colonies were *L. paracasei*, a popular starter culture bacteria in cheese industry and which act as an probiotic strain (Desmond et al., 2004; Smokvina et al., 2013).

## 5.4 MALDI ToF MS analyses for colonies in aerobic conditions

Principal component analysis (Figure 2) shows that both cheese A-good and A-bad are same as there was no colony growth in both cheese types under aerobic conditions. *L. pseudomesenteroide* presented only in cheese C. Starrenburg and Hugenholtz (1991) have explained that this species contributes for the flavor. Also, Hemme and Foucaud-Scheunemann (2004) have mentioned that *L. pseudomesenteroide* is involved in the process of carbon dioxide production and by that giving the characteristic eyes to the round-eyed cheese. This is in agreement with the type of cheese C. Cheese B was consisted with both *L. paracasei* and *L. lactis* which are one of the most popular starter culture bacteria in cheese production. Although, *L. paracasei* does not favor on aerobic conditions, some growth could be seen in cheese B in aerobic conditions. Possibly this could be a subspecies or strain which can tolerate the aerobic conditions. This is in agreement with Cerbo and Palmieri (2013) who have reviewed that although *Lactobacilli* are anaerobic, they can tolerate aerobic conditions. However, these microorganisms need to be identified in terms of sub species or strains level as further investigations.

## 6 Conclusions

The log cfu/g of cheese was not significantly different among cheese A-good, A-bad, B and C. However, microbial composition was different for all studied cheese types. Good and bad sensorial cheeses were different in terms of microbial composition. Highest presence of unidentified organisms could be seen in cheese A-bad due to the limited database of MALDI ToF MS. However, since the aim of the current study was to get an overall picture of microbial composition of different cheese, MALDI ToF method can be concluded to be a suitable one because of its rapidity and accurate results without complicated laboratory procedures. However, for identification in sub species or strain levels, further investigations should be done with DNA or RNA based analyzing methods.

## 7 Future perspectives

Some of the new researches can be created based on this research to clarify the emerged problematic aspects as listed below:

- MALDI ToF MS can be improved to identify subspecies or strain levels by improving its database with details of spectra of subspecies and strains
- Unidentified organisms can be identified by DNA or RNA based molecular methods to test whether there was any linkage between these organisms and sensorial properties

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## 10 Appendix 1

### Microbial growth in different agar media in anaerobic and aerobic conditions

*L. paracasei*, *L. lactis* and *P. freudenreichii* were identified in anaerobic conditions. All three microorganisms were observed in TSA media. *L. paracasei*, *L. lactis* and *L. pseudomesenteroide* were identified in aerobic conditions. All three microorganisms could be detected in rogosa medium. The species grown in M-17 and PCA media were *L. lactis* and *L. paracasei*.

Table 1. Presence of different microorganisms in different media under aerobic and anaerobic conditions

Microorganism	Medium			
	Rogosa	M-17	PCA	TSA
<i>Lactobacillus paracasei</i>	AB	B	B	B
<i>Lactococcus lactis</i>	AB	AB	AB	B
<i>Propionibacterium freudenreichii</i>	NG	NG	NG	B
<i>Leuconostoc pseudomesenteroide</i>	A	NG	NG	NG
Unidentified organisms	A	NG	B	AB

AB-Grow under both aerobic and anaerobic conditions

B-Grow under anaerobic conditions only

A-Grow under aerobic conditions only

NG-Not growing in any condition

Shape of the colonies in all four media was round and greyish white in colour. However, the colonies grown in M-17 plates were larger than colonies grown on other media. The colonies grown in rogosa and TSA were very small and higher in density.

According to the Table 1, *L. lactis* and *L. paracasei* have grown in all four media; rogosa, M-17, PCA and TSA. Since TSA is a general type of medium most of

the microbiota has grown on these plates. An exception was *P. freudenreichii*. Therefore, TSA can be used as a good medium to identify microorganisms present in the cheese.