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Microbial processes and volatile metabolites in cheese

detection of bacteria using an electronic nose

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Abstract

Cheese is a fermented product in which bacteria contribute to different flavours and textures. In order to understand the microbial processes in cheese, it is necessary to not only look at the genomic information in bacteria. The metabolome consists of a complete collection of metabolites in a biological sample. These metabolites are small molecules with a M_r >1.5 kDa, including flavour compounds. During the ripening process of cheese, many microbiological and biochemical changes occur that give cheese a diversity of textures and flavours. Proteins that go through proteolysis and amino acid catabolism are of great importance in the development of flavour in cheese, regardless of variety. Even though techniques for measurements of metabolites have existed for a long time, there are some unique challenges by analysing of several metabolites in parallel in a biological sample that promotes different metabolic pathways. Metabolic fingerprinting is the most common approach used in metabolomics, which is based on statistical analysis that through algorithms presents differences between samples. The electronic nose is able to identify the sum of volatile metabolites in a food, which is unlike the gas chromatograph that identifies individual metabolites. The aim of this review is to evaluate the use of metabolomics of selected *Enterobacteriaceae* together with electronic nose technology in order to analyse possible patterns of volatile metabolites produced in soft cheese. By this we hope to evaluate potential application of this approach in food quality control and microbial contamination screening. The pilot study was done together with the center for AASS, Örebro University where bacteria were analysed using the electronic nose NST3320. The study showed that it is possible to discriminate between *Enterobacteriaceae, Staphylococcus aureus* and cheese-associated bacteria, but also between the *Enterobacteriaceae* species *Escherichia coli, Hafnia alvei* and *Klebsiella pneumoniae*. It is important to consider the gas sensors gradually lose their ability to detect substances after continual use, in which they need to be replaced with new gas sensors. Further, data processing requires special knowledge and can be hard to handle if the expertise is lacking. We believe that there is evidence that metabolomics together with the electronic nose have future prospects in terms of quality control and microbial contamination screening.

Keywords: *Enterobacteriaceae, Lactococcus,* electronic nose.

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1 Introduction

Bacteria contribute to different flavours and textures in cheeses during ripening. Numerous reviews have made important scientific contributions with regards to the production of volatile metabolites by bacteria in cheese; these metabolites vary considerably between bacterial isolates and is strain dependent (McSweeney and Sousa, 2000; Curioni and Bosset, 2002; Bos et al., 2013; Steele et al., 2013). Moreover, cheese contains citrate, lactose, triglyceride and casein that promote different metabolic processes in the production of volatile metabolites (Marilley and Casey, 2003). In order to understand the microbial processes in cheese, it is necessary to not just look at the genomic information in bacteria. Post-genome science, especially metabolomics, has the potential to be more dynamic in different physiological and environmental states (Vaidyanathan, 2005).

Arribas (2013) explains metabolomics as the comprehensive analysis of metabolites present in a biological sample (metabolome). Metabolomics can be used differently, one of these approaches have been called ‘fingerprints’ which gives a chemical profile of the sample tested (Le Boucher et al., 2013). This is an untargeted approach that requires no previous knowledge of present metabolites (Le Boucher et al., 2013). Ochi et al. (2012) have used the ‘fingerprint’ approach and showed that it was possible to predict the sensory characteristics “rich flavour” and “sour flavour” by analysing a number of metabolites (including amino acids, fatty acids, amines, organic acids, and saccharides) in cheese using gas chromatography/time-of-flight mass spectrometry. The authors conclude that metabolomics represents a major advance towards understanding cheese quality (Ochi et al., 2012). Another example is a study by Pogačić et al. (2015) where they used a metabolomics-based method of data processing to evaluate different genera of cheese-associated bacteria for their ability to produce volatile metabolites. The authors conclude that this approach can be used to evaluate cheese-associated bacteria for their potential to enhance the flavour of semi-hard cheeses (Pogačić et al., 2015). Putri et al. (2013) contributes with a comprehensive review of studies that have used metabolomics for quality control and safety.

Before metabolomics can be effectively applied, methodological issues such as sampling, statistical analysis and communication of results should be established (Arribas, 2013). Even though techniques for measurements of metabolites have existed for a long time, there are some unique challenges by analysing of several metabolites in parallel in a biological sample that promotes different metabolic pathways. Therefore, conventional analysis techniques such as gas

chromatography will have a problem capturing the complexity of most food aromas. Sensory analysis using the human nose can be an option but there will always be variability between individuals, which can give inconsistent results, especially when analysing small differences between samples (Lawless and Heymann, 2010). An electronic nose is similar to a human nose because it identifies the sum of volatile metabolites, unlike the gas chromatography that identifies individual metabolites. Metabolomics appears to be theoretically as a good complement to the electronic nose technology since metabolomics is a comprehensive analysis of metabolites present in a biological sample, such as cheese. Previous studies have successfully "taught" the electronic nose to discriminate between bacteria (Gibson et al., 1997; Dutta et al., 2002; Ali et al., 2003; Marilley et al., 2004; Moens et al., 2006; Trincavelli et al., 2010; Green et al., 2011; Gobbi et al., 2014). According to Loutfi et al. (2015), electronic nose technology developed notable achievements during the past few years that could make is relevant for the food industry.

In my previous studies, moderate correlations between the amount of *Enterobacteriaceae* and the intensity of the sensory characteristics "bitter", metallic", "manure" and "pungent" have been presented (Westling, 2013; Westling, 2014). The results indicated that it was possible to detect high levels of *Enterobacteriaceae* in soft cheeses made from raw milk using only the human senses (odor and taste). It was not possible to see whether there were any different sensory profiles between *Enterobacteriaceae* species. According to other studies, *Enterobacteriaceae* species have the ability to decarboxylate both lysine and ornithine with the result that the biogenic amines cadaverine and putrescine are produced (Marino et al., 2000; Durlu-Özkaya et al., 2001; Chaves-López et al., 2006; Pircher et al., 2007). This was not the general case for other bacteria (enterococci, *Lactobacillus* and *Leuconostoc*) tested in these studies. Cadaverine and putrescine have distinctive smells which relates to the sensory characteristic "pungent" and possibly also "bitter", "metallic" and "manure". According to Pircher et al. (2007) there are, however, substantial intra-species differences in putrescine and cadaverine formation. Perhaps, an interdisciplinary approach with metabolomics and electronic nose technology can provide new insight into the impact that bacteria has on the sensory characteristics in cheeses during ripening.

1.1 Aim and objective

The aim of this review is to evaluate the use of metabolomics of selected *Enterobacteriaceae* together with electronic nose technology in order to analyse possible patterns of volatile metabolites

produced in soft cheeses. By this we hope to evaluate potential application of this approach in food quality control and microbial contamination screening. The objectives are (1) to sum previous research that have used the electronic nose to monitor bacteria and (2) to test whether *Enterobacteriaceae* species can be distinguishable using the electronic nose technology.

2 Microbiology in cheese production

Cheese is made from milk that has been coagulated either by lowering pH or by adding rennet, a complex enzyme mixture from the stomach of animals (Kindstedt, 2013). During coagulation, the milk is separated into solids containing protein and fat that combine to form curds, and liquids including water and lactose to form whey (Kindstedt, 2013). The curds are then drained and pressed to expel the whey, usually ranging from 30 to 60%, depending on the type of cheese (Kindstedt, 2013). During this process, the acidification and demineralisation is controlled as well as the salt content, that varies from 0,5% to 4,6% or higher (Kindstedt, 2013). During the ripening process, microbes ferment the carbohydrates in the cheese. Starter culture is added before the milk gets separated, usually consisting of one or more different lactic acid bacterial (LAB) strains (Kindstedt, 2013). When the milk is not pasteurised, an undefined variety of bacteria including contaminants may be present within cheese, and their concentration changes over time, from manufacture to its consumption (Le Boucher et al., 2013). The activity of these cheese-associated bacteria and possible contaminants during the ripening process gives the cheese a diversity of textures and flavours, especially those made from raw milk (Le Boucher et al., 2013).

2.1 Cheese-associated bacteria and contaminants

2.1.1 Bacteria associated with cheese production

Microorganisms may contaminate cheese during production, including preparation, ripening and handling, therefore, steps are taken to minimise this problem. The use of extremely high quality milk in combination with good hygiene practices is not enough to ensure a cheese without contaminants. Neither is the use of 60-days maturation enough to ensure a cheese without contaminants. In order to control the microbial content, milk can be pasteurised before it is used to make cheese.

Pasteurisation kills different microbes that may be present in foods such as milk. In Sweden, low pasteurised milk is heated at 72-74°C for 15 seconds and high pasteurised milk at 80°C for five

seconds. Low pasteurised milk may still contain bacterial species such as *Bacillus, Clostridium,* coryneform bacteria, micrococci, *Streptococcus thermophilus* and enterococci (Svensk mjölk, 1998; 2003). Furthermore, enzymes produced by psychrotrophic bacteria and bacteriophages remain active (ibid.). In high pasteurised milk, only spore-forming bacteria survive and some enzymes remain active (Svensk mjölk, 1998). Unpasteurised milk may contain bacteria such as *Campylobacter, Salmonella, Escherichia coli, Listeria monocytogenes, Yersinia enterocolitica, Staphylococcus aureus, Mycobacterium bovis, Brucella* and *Coxiella burnetti* that may cause food-borne diseases (Livsmedelsverket, 2012). For example, coagulase-positive staphylococci are important contaminants often found in raw milk and some strains form heat-stable enterotoxins that can cause nausea, vomiting, abdominal cramps, diarrhea, headache and hypotension (Livsmedelsverket, 2012). *L. monocytogenes* is another important contaminant found in raw milk and it has the ability to adapt to different environments so that it can grow at low temperatures down to 0°C and in low water activity (Livsmedelsverket, 2012). Invasive listeriosis is a rare disease that can be life threatening with a mortality rate of 20-30% (Livsmedelsverket, 2012). Most cases of listeriosis are found in the immunocompromised people, elderly and pregnant woman (Livsmedelsverket, 2012). It is therefore important to control these pathogens during cheese production.

To limit the amount of potential pathogens in the cheese process, the use of starter cultures that compete with contaminants is a good complement to pasteurisation but may also be an alternative to the pasteurisation of milk. LAB are often used as starter cultures in cheese production, which deliver a more predictable rate of acidification (Kindstedt, 2013). Starters in cheese production include *Lactococcus lactis, Lactobacillus helveticus* and *Streptococcus thermophiles* (Kindstedt, 2013). But in order to prevent a high content of contaminants during cheese ripening, particularly of cheese made with raw milk, monitoring is required (Özoğul, 2004). It is therefore not sufficient to depend on different steps to minimise the contamination of cheese, especially not when the cheese is sold on a market.

2.1.2 Monitoring contaminants in cheese ripening

Conventional methods such as plate counting are still used to monitor contaminants in cheese. However, plate counting usually requires a minimum of 24 hours for bacteria to grow to sufficient numbers to be enumerated. Several researchers have discussed the family *Enterobacteriaceae* (to which *E. coli* belongs) as a hygiene indicator in cheese (Giammanco et al., 2011; Chaves-López et

al., 2006). Since several species within this family occur normally in many different foods, it can sometimes be difficult to draw any conclusions about the cheese's hygienic condition based on this parameter. Despite this, Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs states that *Enterobacteriaceae* can be used as a risk indicator for routine monitoring in both the manufacturing environment and in the final product (Europakommissionen, 2005). The same regulation states that food business operators should be able to use analytical methods other than the reference methods, particularly rapid methods, provided that the use of these alternative methods yield comparable results.

2.2 Metabolic processes influencing cheese sensory characteristics

During the ripening process of cheese, many microbiological and biochemical changes occur that give cheese a diversity of textures and flavours. The development of texture and flavours is a dynamic process that is impacted by the type and composition of milk, in particular due to the microorganisms and enzymes. According to Marilley et al. (2004), this development of texture and flavour is mainly carried out by cheese microflora. The same authors have summarised the biochemical pathways leading to the development of the flavour compounds in cheese (see *Fig. 1*).

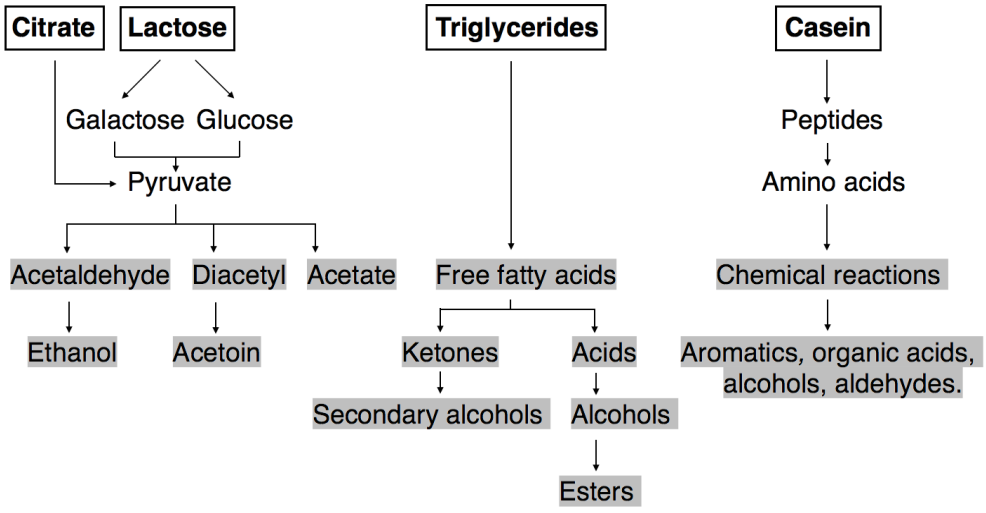

Fig. 1. General overview of the biochemical pathways leading to the formation of flavour compounds. The grey surface indicates flavour compounds (figure adapted from Marilley and Casey, 2003).

The substrates enabling these pathways are organic acids (citrate), carbohydrates (lactose), lipids (triglycerides) and proteins (caseins). Of these substrates, proteins that go through proteolysis and

amino acid catabolism are of great importance in the development of flavour in cheese, regardless of variety (Steele et al., 2013). Numerous studies have shown that the production of volatile compounds varies considerably between bacterial isolates and is strain dependent (McSweeney and Sousa, 2000; Curioni and Bosset, 2002; Bos et al., 2013; Steele et al., 2013). Cheese made from raw milk is therefore particularly complex due to the varying composition of microbes (Hassan et al., 2013; Montel et al., 2014). To go into detail of the formation of these flavour compounds due to metabolism during ripening, they may be grouped into primary and secondary metabolic pathways.

2.2.1 Primary metabolic pathways

The primary metabolic pathways include lipolysis, proteolysis and metabolism of residual lactose and of lactate and citrate (McSweeney, 2004). Free fatty acids result from lipolysis, which is catalysed by lipase from various sources, such as bacteria and by enzymes from rennet, when this coagulant is used (McSweeney, 2004). Free fatty acids can be precursors to flavour compounds such as ketones, acids, alcohols and esters (Marilley and Casey, 2003). These flavours are especially important in soft cheeses, e.g. Camembert and Roquefort (Smit et al., 2005).

Peptides and free amino acids results from proteolysis which is catalysed by enzymes from residual coagulant, the milk and bacteria (McSweeney, 2004). Proteolysis is in fact the most complex metabolic pathway during cheese ripening (McSweeney, 2004). However, peptides and amino acids do not generally impact the flavour characteristics in cheese, other than the basic tastes such as bitter (Smit et al., 2005). Further conversion of amino acids to various aromatics, organic acids, alcohols and aldehydes is required for flavour formation (Marilley and Casey, 2003). In matured cheeses, starter culture die relatively quickly and this influences the rate at which free amino acids are formed (Urbach, 1995).

Metabolism of residual lactose and of lactate and citrate is a rapid process during the early stages of ripening (McSweeney, 2004). Not surprisingly, lactic acid bacteria contribute with the primary formation of lactate. Further conversion from the intermediate pyruvate can contribute to various flavour compounds such as acetaldehyde, diacetyl, acetoin and acetate (Marilley and Casey, 2003). These flavours are especially important in yoghurt, some of which contribute to typical yoghurt flavours (Smit et al., 2005).

2.2.2 Secondary metabolic pathways

The secondary metabolic pathways include metabolism of fatty acids and of amino acids (McSweeney, 2004). Amino acids are degraded through transamination reactions and various flavour compounds are then produced from one or two additional reactions (Yvon and Rijnen, 2001). The flavour compounds resulting from this process have significant impact on the resulting flavour in cheese (Steele et al., 2013). For example, some acids can contribute with the sensory characteristics “rancid”, “putrid” and “sour”, which contributes to the perception of ripened cheese (Yvon and Rijnen, 2001).

3 Metabolomics

The metabolome consists of a complete collection of metabolites in a biological sample (Arribas, 2013). These metabolites are small molecules with a M_r >1.5 kDa, including flavour compounds mentioned in *Fig. 1*. As mentioned in the introduction, metabolic fingerprinting is the most common approach used in metabolomics (Arribas, 2013). This technique can be used to classify samples, which is based on statistical analysis that through algorithms present differences between samples (Arribas, 2013). This approach does not require previous knowledge of the metabolites present in the metabolome at the same time as it allows a quick classification of samples (Le Boucher et al., 2013). There are also targeted approaches that require previous knowledge of the metabolites in addition to an initial hypotheses (Le Boucher et al., 2013). These approaches often uses a combination of techniques; a common combination is gas chromatography with mass spectrometry (GC-MS). For example, a study by Pogačić et al. (2015) used a headspace trap method coupled to a GC-MS and a metabolomics-based method of data processing, with statistical and multivariate analyses. The authors conclude that this approach can be applied to evaluate cheese-associated bacteria for their ability to produce flavour compounds and particularly for their potential to enhance the flavour of semi-hard cheeses. But the authors also conclude that this targeted approach can be very time consuming when there are many samples to compare. Another example is a study by Ochi et al. (2012) who revealed that metabolomics-based component profiling was able to predict the sensory characteristics of cheese related to ripening, by focusing on metabolites. Thereby, the authors demonstrate that sensory results can be combined with the metabolome profile, using multivariate analyses. Overall, metabolomics contributes with advances in quality control because it can evaluate multiple factors simultaneously (Putri et al., 2013).

3.1 Flavour profile of cheese-associated bacteria and contaminants

Flavour perceptions are a complex set of reactions. For example, Niimi et al. (2014) shows that the perceived intensity of the "cheese flavour" was enhanced by sucrose and NaCl, while it was suppressed by lactic acid. This is due to sensory interactions between taste and aromas (Niimi et al., 2014). According to Lawless and Heymann (2010), machines, instead of humans, can be used when a correlation between sensory results and data derived from instruments has been established. Machines can also be used when sensory tests danger panelists' health (Lawless and Heymann, 2010). My previous studies have presented correlations between *Enterobacteriaceae* and sensory results (Westling, 2013; Westling, 2014). Furthermore, *Enterobacteriaceae* may be harmful to the consumer (Tham and Danielsson-Tham, 2014). Therefore, it is relevant from both points of views to evaluate the use of other instruments then humans.

A combination of flavour compounds when compared with individual flavour compounds may yield different flavours than those expected. Chambers and Koppels (2013) review addresses issues in association sensory results with other data, such as chemicals. The authors state that it is important to use carefully crafted semantic lexicons when comparing sensory results with other data. In my previous studies the semantic words "bitter", "metallic", "manure" and "pungent" were used (Westling, 2013; Westling, 2014). The perceived intensity of these semantic words were correlated with the amount of *Enterobacteriaceae* 37°C in soft cheeses (ibid.). This may at least decrease the confusion when trying to compare the sensory results with the metabolites present in cheese. *Enterobacteriaceae* will be discussed below with regards to flavour profiles. Also, *Lactococcus lactis* is discussed because it is the most common used LAB in cheese production (Kindstedt, 2013).

3.2.1 *Enterobacteriaceae* species

Durlu-Özkaya et al. (2001) concluded that putrescine, cadaverine, tyramine and histamine are produced by *Enterobacteriaceae,* both in culture medium and in meat products. Also, other authors have shown that *Enterobacteriaceae* can produce putrescine and cadaverine (Marino et al., 2000; Chaves-López et al., 2006; Pircher et al., 2007). Pircher et al. (2007) demonstrated that none of the 137 tested enterococci and only two isolates of 251 *Lactobacillus* and *Leuconostoc* isolates tested produced >100 mg/l of cadaverine or putrescine. In contrast, 147 of 149 isolates of *Enterobacteriaceae* was capable of forming >100 mg/l of either cadaverine or putrescine (Pircher et al., 2007). According to Pircher et al. (2007) there are, however, substantial intra-species differences

in putrescine and cadaverine formation.

Elgaali et al. (2002) have shown that the emission patterns of volatile compounds from bacteria associate long chain alcohols (1-octanol, 1-decanol and 1-dodecanol) with enteric Gram negative bacteria, which included *Citrobacter, Enterobacter, Klebsiella, Salmonella* and *Shigella*. The long chain alcohols were not detected as products from the non-enteric Gram negative species studies, which included *Acinetobacter, Pseudomonas,* and *Shewanella* (Elgaali et al., 2002). Among Gram positive bacteria, including *Bacillus, Enterococcus, Lactococcus, Leuconostoc, Listeria, Staphylococcus,* and *Streptococcus*, the only long chain alcohol detected was 1-decanol and, when present, it occurred in relatively small amounts (Elgaali et al., 2002). Hamilton-Kemp et al. (2005) have also examined the production of 1-octanol, 1-decanol and 1-dodecanol of 10 strains of *E. coli* in tryptic soy broth for volatile metabolites using solid-phase microextraction. Long-chain alcohols were produced by all strains, which followed the onset of the exponential growth phase of the broth culture (Hamilton-Kemp et al., 2005). The authors discusses that these neutral volatile alcohols may have application as vapour-phase indicators for certain classes of bacteria, particularly Gram negative enteric bacteria. Maddula et al. (2009) used a multi capillary column coupled to an ion mobility spectrometry to detect volatile metabolites of *Escherichia coli.* Four analytes correlated positively with growth, which were identified as ethanol, propane, heptan-2-one and nonan-2-one (Maddula et al., 2009).

In a study by Maharjan and Ferenci (2005) it was shown that no two *E. coli* isolates had the same metabolome profile. In the same study, the metabolome patterns did not cluster in relation to the pathogenicity or environmental origins of strain, although unique metabolite spots were found in most bacteria (Maharjan and Ferenci, 2005). This indicates that metabolic diversity is likely to be characteristic of a bacterial species (Maharjan and Ferenci, 2005).

3.2.2 *Lactococcus lactis*

According to Urbach (1995), the main contributions of LAB in the flavour of fresh cheeses are the conversion of lactose to lactic acid and the production of diacetyl and acetaldehyde. LAB contribute relatively little to lipolysis (Smit et al., 2005). It has been shown that the abilities of proteolysis differ between various LAB strains (Smit et al., 2005). Generally, wild *L. lactis* strains require generally only two or three amino acids to grow while industrial strains require around seven or more amino acids to grow, such as glutamate, valine, methionine, histidine, serine, leucine and

isoleucine (Smit et al., 2005). This gives wild *Lactococci* a larger potential in proteolysis and consequently produce other, rather unusual, flavour compounds, that give the cheese other flavour profiles (Smit et al., 2005). But these strains could lack other enzymatic activities, such as fast acidification (Smit et al., 2005). According to Smit et al. (2005) it is possible to combine wild and industrial strains in order to obtain both good flavour as well as good acidifying and proteolytic activities. Furthermore, in a study by Ziadi et al. (2008), it was shown that a mixture of *L. lactis* strains resulted in higher production of volatile compounds than single strain cultures (Ziadi et al., 2008). A mixture of strains can in fact complement each other in this formation of flavour compounds, which is demonstrated by Ayad et al. (1999) who used GC-MS in combination with sensory evaluation. Additionally, in a study by Gutiérrez-Méndez et al. (2008), an aroma potentiation effect was demonstrated when using a mixture of two lactococci strains from different sources. Yvon and Rijnen (2001) states that several LAB, including *L. lactis,* are capable of degrading methionine to methanethiol, which have been associated with the sensory characteristic "sulfur" and "garlic" in cheese. In a study by Azizan et al. (2012), it was observed that *L. lactis* produced more organic acids under agitation at 150 rpm compared with no agitation. Therefore, both internal and external factors may contribute to the resulting flavour compounds present in cheese.

4 Electronic nose technology

The electronic nose uses gas sensors to analyse gases in the environment. When analysing food samples, head-space sampling is a common practice to detect volatile compounds (Chambers and Koppel, 2013). This is done by using closed chambers, such as vials, where as a needle penetrates the lid and analyses the gases from the head-space. One of the most important characteristics of the electronic nose technology is that it does not identify individual compounds but the sum of these (Chambers and Koppel, 2013). In order to get valuable results, the large data collected needs to be processed through statistical analysis. Common statistical methods used with the electronic nose technology is principal component analysis (PCA), linear discriminate analysis (LDA), partial least squares (PLS), functional discriminate analysis (FDA) and cluster analysis (CA) (Loutfi et al., 2015). The results obtained from these methods can be used to identify relationships, such as grouping or spreading of data within samples. Because electronic noses detect a wide range of gases, algorithms are used to extract relevant information (Boeker, 2014). This process is called artificial intelligence, a process that enables the electronic nose to detect the compounds of interest.

4.1 Monitoring microbes using an electronic nose

Electronic noses are becoming more popular to identify sensory characteristics of different foods because they are relatively easy to build, cost-effective and provide a short time of analysis (Escuder-Gilabert, 2009). The use of the electronic nose for the detection of microbial spoilage in dairy products, such as cheese, is discussed by Magan et al. (2001). The authors suggest that electronic nose technology might provide a useful tool in the future for early detection of spoilage in dairy products (Magan et al., 2001). Additionally, the authors discusses that it might be possible to choose gas sensors that detect volatile metabolites associated with contaminants for quality control in the dairy industry (Magan et al., 2001).

As mentioned in the introduction, previous studies have successfully used the electronic nose to discriminate between bacteria (Gibson et al., 1997; Dutta et al., 2002; Ali et al., 2003; Marilley et al., 2004; Moens et al., 2006; Trincavelli et al., 2010; Green et al., 2011; Gobbi et al., 2014). In a study by Ochi et al. (2012), PLS regression models were constructed to predict the relationship between the metabolite profile and two sensory characteristics that were related to maturation, namely “rich flavour” and “sour flavour”. This study (ibid.) concludes that it is possible to predict sensory characteristics in cheese by focusing on low molecular weight compounds with a metabolomics-based component profiling. In a study by Green et al. (2014) the results demonstrated classification accuracies greater then 80% when identifying single colonies of bacteria at the genus level with the electronic nose. Further, it was shown that higher classification accuracy (96,7%) could be achieved by repeated sampling of the same colony and using all available odor responses to characterise a sample (Green et al., 2014). In a study by Statham Thorn et al. (2011), it was shown that there are significant differences in the volatile profiles obtained from various bacterial monocultures grown in vitro. It was concluded that the use of multivariate analysis using similarity matrices, cluster analysis and multidimensional scaling have the potential to differentiate samples at the strain level (Statham Thorn et al., 2011).

To ensure quality and safety in food products such as cheese, fast and precise methods are necessary (Gobbi et al., 2014). According to Gobbi et al. (2014), thus the electronic nose includes both good identification of metabolites and the potential of correlating data with that of the microbiological tests and sensory evaluations. Additionally, the electronic noses can be portable and used in the

production processes, and once trained these machines can work standalone (Gobbi et al., 2014; Loutfi et al., 2015). Loutfi et al. (2015) states that despite these features, there are relatively few electronic noses used in the industry. According to Green et al. (2014), the electronic nose holds the potential to shorten the testing times of bacteria and thereby could complement existing methods used to monitor contaminants. In the study by Eklöv et al. (1998) the authors showed that the electronic nose has great possibilities in the area of quality control and process monitoring in the meat industry. In the dairy industry, Ali et al. (2003) states that the electronic nose can be used in quality control and monitoring of off-flavours during milk storage.

5 Discriminate bacteria using electronic nose – a pilot study

5.1 Introduction

Detection and identification of bacteria using electronic nose technology have been successful in previous studies (Gibson et al., 1997; Dutta et al., 2002; Ali et al., 2003; Marilley et al., 2004; Moens et al., 2006; Trincavelli et al., 2010; Green et al., 2011; Gobbi et al., 2014). Some members of the family *Enterobacteriaceae* can be pathogenic and have been found in cheeses, for example *H. alvei, E. coli* and *K. pneumoniae.* Also, *S. aureus*, a common skin bacteria, can be found in cheese and produce enterotoxins. All of these bacteria are contaminants and should be controlled during cheese production. *L. lactis* is the most common LAB in cheese production (Kindstedt, 2013). In this pilot study, *H. alvei, E. coli, K. pneumoniae, S. aureus* and *L. lactis* will be added separately to milk powder broth and agar and analysed using head-space sampling with electronic nose technology. The electronic nose NST3320 with Metal-Oxide-Silicon Field-Effect-Transistor (MOSFET) gas sensors will be used to see if it is possible to discriminate between bacteria, both between *Enterobacteriaceae* species and these species against *L. lactis* and *S. aureus.* The same electronic nose, NST3320, has successfully been used by Trincavelli et al. (2010) to discriminated bacteria in blood samples.

5.2 Methods

5.2.1 Bacterial species and growth conditions

Three *Enterobacteriaceae* species *H. alvei, E. coli* and *K. pneumoniae* were collected during my previous studies (Westling, 2013; Westling, 2014). These bacteria were originally collected from French soft cheeses, bought from a cheese store in Sweden. *L. lactis* was obtained from the centre for Life Science, Örebro University and *S. aureus* was obtained from campus Grythyttan, Örebro

University. *Enterobacteriaceae* species and *S. aureus* were removed from -80°C storage and cultured aerobically on blood agar plates, while *L. lactis* was cultured anaerobically on M17 agar plates. The cultivation of bacteria isolates was done using a three-phase-streak-plating technique. All bacteria were incubated at 37°C for 36-48 h. After incubation, bacteria was moved to a 4°C storage.

5.2.2 Preparation of culture media for electronic nose analysis

A milk mixture was made by combining 20 g of milk powder to 100 ml water in a conical flask, which was boiled using a magnetic stirrer with heat for about two minutes until homogeneous. The milk mixture was transferred aseptically into four sterile tubes. One part of milk mixture and nine parts of agar or broth were measured in each tube. Nutrient and M17 agar were used for solid media growth and nutrient and M17 broth was used for liquid cultures. Agar and broth were dispensed in 2 ml volumes into vials constructed for the electronic nose NST3320. The vials with agar were set at room temperature to result in agar slants. A total of 12 vials were used. Single colonies were picked from each inoculation source and were streaked onto the surface of the agar medium and into the milk containing broth. M17 agar and broth were used for *L. lactis* while the nutrient medium was used for the other bacteria. Also, two media blanks were used (one nutrient broth and one M17 broth). All vials were incubated for 40 h at 37°C. Order and safety regulations were followed in microbiological laboratory.

5.2.3 Sampling procedure of selected bacteria using an electronic nose

The bacterial cultures were taken to the centre for AASS in closed vials and analysed using the electronic nose. Gases from the head-space in each vial were analysed automatically with the electronic nose NST3320. The electronic nose was calibrated to analyse each vial five times for five minutes each, which gave a total of 60 readings (five for each of the twelve classes) and a length for data collection of five hours. The electronic nose was also calibrated for 30°C during the incubation and room temperature during the standby.

5.2.4 Statistical analysis

The statistical analysis of the data collected was done by Amy Loutfi and Martin Längkvist at the centre for AASS, Örebro university. The data was processed with a Linear Discriminant Analysis (LDA). Also, four classifiers were tried: Softmax, Naïve bayes, linear Support Vector Machine (SVM) and non-linear SVM.

5.3 Results and discussion

The cheese-associated bacteria *L. lactis* and the contaminants *H. alvei, E. coli, K. pneumoniae* (*Enterobacetriaceae*) and *S. aureus* were added to the milk powder agar and broth. After incubation, gases from the head-space were analysed with the electronic nose NST3320. The collected data was processed with a LDA and four classifiers. In the LDA of selected bacteria using an electronic nose it can be seen that all of the readings of the same bacterial species are close together, regardless of the culture medium used (*Fig. 2*). It can also be seen that the blank nutrient agar broth is at a distance on the Y-axis from all the others, indicating that it is the bacteria which contributed to the statistical differences between samples. It can be seen on the X-axis that all *Enterobacteriaceae* species in both agar and broth are relatively close together, while *S. aureus, L. lactis* and M17 broth are farther away. M17 broth is very close to *L. lactis,* which is reasonable since *L. lactis* was grown on M17 broth. In contrast to nutrient broth, M17 broth has a distinctive smell that implies it contains gases that were detected by the electronic nose. Overall, these results can be compared with results from Marlier et al. (2004) who demonstrated that lactic acid bacteria with the same genotype clustered when using an electronic nose. Gobbi et al. (2014) used LDA for classification performance and achieved a performance of 98% when measuring *Enterobacteriaceae* in vegetable soups with different incubation times.

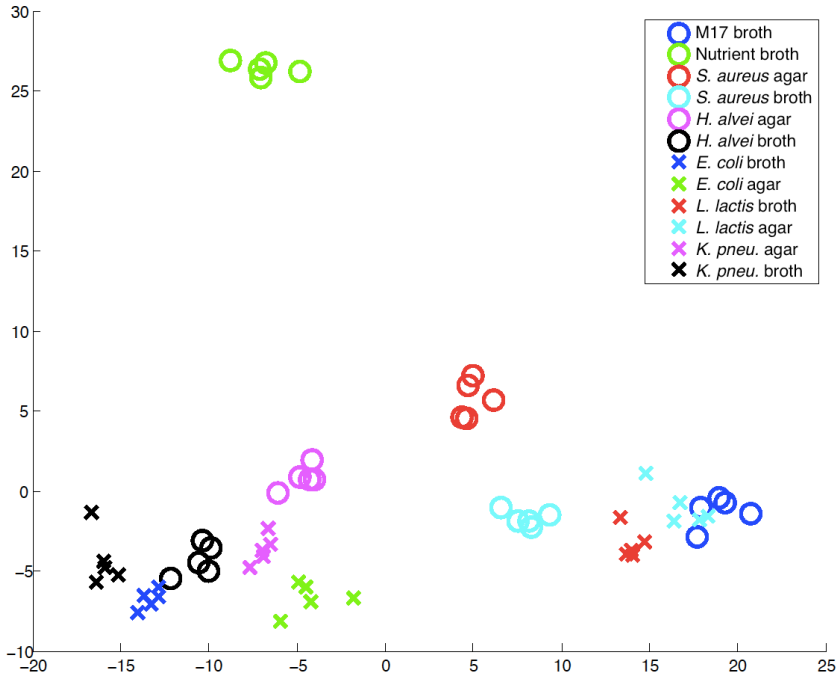

Fig. 2. Linear discriminant analysis of selected bacteria using an electronic nose. O and X in different colours refers to the different bacteria grown on agar slants and in liquid broth. Also, two blanks are included (M17 broth and nutrient broth).

To determine if the electronic nose was able to discriminate between the different bacteria and growth media, four classifiers where tried. These were Softmax, Naïve bayes, linear SVM, non-linear SVM. The classification accuracy was for Softmax 58,33%, for Naïve bayes 31,67%, for linear SVM 43,33% and for non-linear SVM 58,33%. Since the data set is so small a full leave-one-out cross-validation is performed, meaning that one reading is used to evaluate the classifiers and the 59 other readings are used to train the classifiers. This is repeated 60 times until all readings have been tested. The best classifiers are Softmax and non-linear SVM that both classified 35 of 60 readings correctly. A confusion matrix with Softmax classifier of selected bacteria using an electronic nose is presented in *Table 1*. Softmax classifier is a pattern recognition algorithm that maps input data to categories. The performance of the algorithm is visualised with a confusion matrix. In this confusion matrix it can be seen if it is possible to distinguish between the different samples.

		Predicted as											
		M17 broth	Nutrient broth	*S. aureus* agar	*S. aureus* broth	*H. alvei* agar	*H. alvei* broth	*E. coli* broth	*E. coli* agar	*L. lactis* broth	*L. lactis* agar	*K. pneu.* agar	*K. pneu.* broth
True class	M17 broth	**5**	0	0	0	0	0	0	0	0	0	0	0
	Nutrient broth	0	**4**	0	0	1	0	0	0	0	0	0	0
	S. aureus agar	1	0	**3**	0	0	0	0	0	0	0	1	0
	S. aureus broth	0	0	1	**2**	1	0	0	0	1	0	0	0
	H. alvei agar	0	0	0	1	**2**	0	0	0	0	0	0	2
	H. alvei broth	0	0	0	0	0	**3**	2	0	0	0	0	0
	E. coli broth	0	0	0	0	0	0	**0**	2	0	1	1	1
	E. coli agar	0	0	0	0	0	0	1	**3**	0	1	0	0

		Predicted as											
True class	*L. lactis* broth	0	0	0	1	0	0	0	0	**3**	1	0	0
	L. lactis agar	0	0	0	0	0	0	0	0	1	**4**	0	0
	K. pneu. agar	0	0	0	0	1	0	0	0	0	0	**3**	1
	K. pneu broth	0	0	1	0	0	0	0	0	0	0	1	**3**

Table 1. Confusion matrix with Softmax classifier of selected bacteria using an electronic nose. The numbers are based on the five analyses done for each sample. If the classification accuracy was 100%, it would only be number 5 where *true class* cross *predicted as*. These numbers are in bold.

In a study by Ali et al. (2003) it was possible to discriminate between milk samples and milk samples contaminated with *E. coli,* which was not the case for milk samples contaminated with *Pseudomonas fragi.* In this pilot study, it can be seen that none of the *E. coli* broth was correctly classified but the *E. coli* agar was correctly classified three times. It can also be seen in *Table 1* that the total number of miss-classification for all bacteria that used agar is ten and that used broth is 14. This gives a small indication that perhaps agar is the better medium for these studies. The results show that all five readings of the M17 broth were classified correctly, while the nutrient broth was classified correctly four out of five times. Most miss-classifications of the samples are when the correct bacteria has been identified but the medium is wrong, for example one *L. lactis* agar is classified as *L. lactis* broth and vice versa.

In a study by Trincavelli (2010) with the same electronic nose as used in this pilot study, it was possible to classify bacteria in blood samples with a classification accuracy of 91.8% ± 11.5% and 94% ± 12.7%, respectively. These bacteria included *E. coli, Pseudomonas aeruginosa, S. aureus, Klebsiella oxytoca, Proteus mirabilis, Enterococcus faecalis, Staphylococcus lugdunensis, Pasteurella multocida, Streptococcus pyogenes* and *Hemophilus influenzae* (Trincavelli, 2010). According to Moens et al. (2006), a 100% classification accuracy of clinically important microbes can be achieved by using electronic nose technology. From the results in this pilot study, it appears that the medium may have importance in the classification accuracy. Green (2014) demonstrated

classification accuracies higher than 80% of different bacteria at the genus level, but states that it was possible with a classification accuracy of 96,7% if repeated samplings were used. It is possible that if more replicate analysis of each sample were conducted in this pilot study a higher classification accuracy would be achieved because algorithms generally perform better with a larger data set.

5.4 Conclusion

The statistical analysis of selected bacteria using an electronic nose resulted in one LDA and one confusion matrix with Softmax classifier. *Enterobacteriaceae* species were close to each other in the LDA, indicating that these bacteria are producing similar volatile metabolites. The classification accuracy of bacteria and culture media was 58%, despite the small data set of 60 readings. The algorithms generally perform better with a larger data set. However, a random-guess classifier would only get 8,33% accuracy. Also, the classification accuracy of bacteria, regardless of culture media, would be 71,67%. If the sampling procedure was done for a longer time-period, it is possible that the choice of culture media might play a bigger role for the classification accuracy of the later readings. Because of the small indication that agar can be a better medium then broth during the sampling procedure, the use of agar would be preferred over broth in future studies.

6 General conclusions

Proteins that go through proteolysis and amino acid catabolism are of great importance in the development of flavour in cheese, regardless of variety (Steele et al., 2013). However, peptides and amino acids do not generally impact the flavour characteristics in cheese, other then basic tastes such as bitter (Smit et al., 2005). Further conversion of amino acids to various aromatics, organic acids, alcohols and aldehydes is required for flavour formation (Marilley and Casey, 2003). Numerous authors have shown that *Enterobacteriaceae* can produce putrescine and cadaverine (Marino et al., 2000; Durlu-Özkaya et al., 2001; Chaves-López et al., 2006; Pircher et al., 2007). According to Pircher et al. (2007) there are, however, substantial intra-species differences in putrescine and cadaverine formation. It has been shown that the abilities of proteolysis also differ between various LAB strains (Smit et al., 2005). Wild lactococci has a larger potential in proteolysis and consequently produce other, rather unusual, flavour compounds, which gives cheese other flavour profiles (Smit et al., 2005). Furthermore, in a study by Ziadi et al. (2008), it was shown that a mixture of *L. lactis* strains resulted in higher production of volatile compounds than single strain cultures (Ziadi et al., 2008). In a study by Azizan et al. (2012), it was observed that *L.*

lactis produced more organic acids under agitation at 150 rpm compared with no agitation. Therefore, both internal and external factors may contribute to the resulting flavour compounds present in cheese.

Metabolomics have been shown to contribute with advances in quality control because it can evaluate multiple factors simultaneously (Putri et al., 2013). Putri et al. (2013) discuss that a more practical scientific assessment of metabolomics should be established through technological advances in instrument development and statistical analysis, which the authors call "sensory chemistry". Perhaps it is the combination of metabolomics and electronic nose technology that will close this gap between theory and practice. According to Peris and Escuder-Gilabert (2009) there is, however, much research to be done in sensors technology, statistical analysis, interpretation of results and validation. Boeker (2014) discusses a problem with the application of electronic noses, namely the lack of information with regards to chemical correlates of the intendent measurement aim and chemical aspects of the electronic nose used for measurement. By using metabolomics in combination with electronic nose technology in order to identify bacteria in cheese, it should possible to both explain and measure the metabolites produced from microbial processes in cheese.

A pilot study was conducted in order to test whether *Enterobacteriaceae* species can be distinguishable using the electronic nose technology, an objective of this project. The electronic nose NST3320 was used to analyse three *Enterobacteriaceae* species, namely *H. alvei, E. coli* and *K. pneumoniae, L. lactis* and *S. aureus.* All bacteria were grown on both milk powder broth and agar. *L. lactis* was grown on M17 agar combined with milk powder. This pilot study was done together with the center for AASS, Örebro University where bacteria were analysed using the electronic nose NST3320. The study showed that it is possible to discriminate between *Enterobacteriaceae, S. aureus* and cheese-associated bacteria, but also between the *Enterobacteriaceae* species *E. coli, H. alvei* and *K. pneumoniae*. It is important to consider the gas sensors gradually lose their ability to detect substances after continual use, in which they need to be replaced with new gas sensors. Further, data processing requires special knowledge and can be hard to handle if the expertise is lacking. We believe that there is evidence that metabolomics together with the electronic nose have future prospects in terms of quality control and microbial contamination screening.

6.1 Future studies

Methodologically, a greater number of cheese-associated bacteria and contaminants should be used in future studies. During the sampling procedure, more analyses (10–20 or more) per vial should be used in order to classify different species with a higher accuracy. For a better understanding and development of this technology in the food industry such as cheese, different bacterial combinations and mixtures would need to be evaluated. Furthermore, an improved understanding of the metabolomics of the relevant bacteria would help with increasing the development of the electronic nose and its practical applications.

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