Microbial Growth in Coffee Grinder and Cold Brewed Coffee Beverage

Mikrobiell tillväxt i kaffekvarn och kallbryggt kaffe

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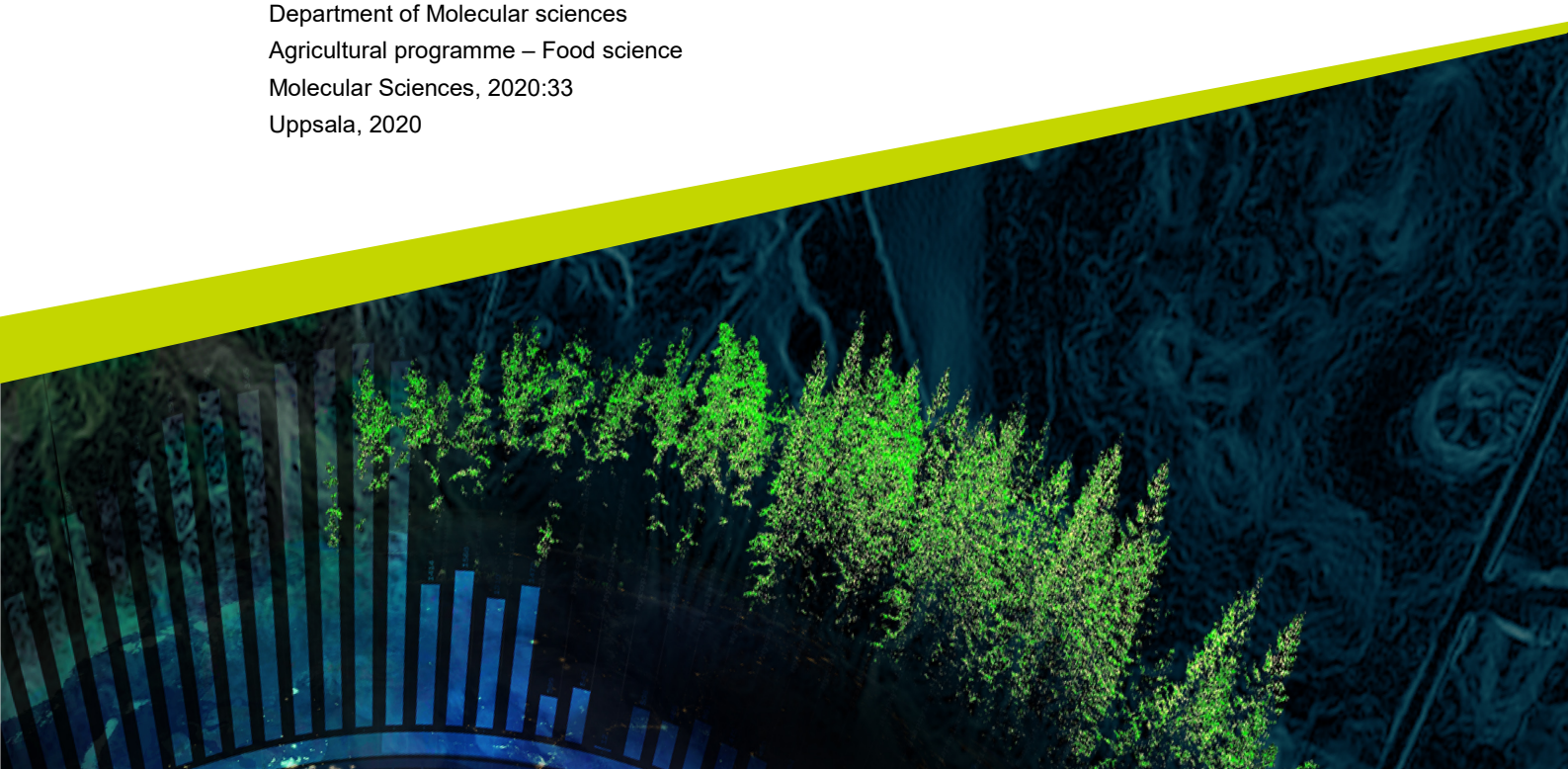

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Abstract

In this project the microbial growth in cold brewed coffee beverage and a well-used coffee grinder was studied. This was a project that was made together with the company 3TEMP ltd in Arvika, Sweden.

The company would like to know how the microbial growth in cold brewed coffee beverage was during different storing conditions. Cold brewed coffee drink is brewed in colder temperatures and usually during longer time to obtain the right aroma and flavour. The storing conditions of the glass flasks was open or closed and in refrigerator (4°C) or in room temperature (25°C). This was obtained by collecting samples from cold brewed coffee beverage and then incubate the samples either open or closed in refrigerator and in room temperature for 3, 4 and 5 and for 1, 3 and 5 days respectively. Samples from the glass flasks were applied to agar plates with selective media, to be able to look at certain groups of microorganisms. Some of the isolates were identified using 16S rDNA or MALDI-TOF. It come to a result of 31 identified bacteria and 2 identified mould samples from those samples run in PCR. From those samples that was run in MALDI-TOF it was 3 identified bacteria.

There was a total of 15 different bacteria and 2 moulds that were identified. The identified bacteria belonged to the genera: *Acinetobacter*, *Bacillus*, *Kocuria*, *Micrococcus*, *Mycobacterium*, *Paenibacillus* and *Staphylococcus*. The identified moulds belonged to *Bjerkandera* and *Neurospora*. The result if those have been found in coffee earlier varied and also if they were considered to be a potential pathogenic microorganism. The most abundant genera that was found belonged to *Bacillus*, whereas some genera were identified once.

Keywords: Microbial growth, Cold brewed coffee, Coffee grinder

Sammanfattning

I denna studie undersöks den mikrobiella tillväxten i kallbryggt kaffe samt en väl använd kaffekvarn. Undersökningen gjordes på företaget 3TEMP ltd i Arvika, Sverige.

Företaget ville ha reda på hur den mikrobiella tillväxten i kallbryggt kaffe är när det kommer till olika förvaringsförhållanden, öppen eller stängd behållare, samt temperatur för förvaring, i kylskåp (4°C) samt vid rumstemperatur (25°C). Detta gjordes genom att samla in prov och inkubera behållarna öppna respektive stängda i kylskåp och i ett rum som håller 25°C. De förvarades i 25°C i 1, 3 och 5 dygn och i 4°C i 3,4 och 5 dygn. Sedan togs prover som odlades på selektiv agar, för att på så sätt kunna undersöka grupper av mikroorganismer. På en del av de plattor där det skett tillväxt gjordes en MALDI-TOF och på alla plattor med tillväxt utfördes PCR och gel-elektrofores för att bestämma om det fanns något som antydde på mikrobiell tillväxt. De prover som visade sig ha tillväxt skickades till Macrogen för identifiering. Totalt skickades 44 prover, varav 42 var bakterieprover och 2 mögelprover. Detta resulterade i att 31 bakterieprover 2 mögelprover kunde identifieras. Från MALDI-TOF identifierades 3 olika bakterier.

Det var totalt 15 olika bakterier som identifierades och 2 olika mögel. De identifierade mikroorganismerna tillhörde släktena*: Acinetobacter*, *Bacillus*, *Kocuria*, *Micrococcus*, *Mycobacterium*, *Paenibacillus* och *Staphylococcus*. De identifierade möglen tillhörde *Bjerkandera* och *Neurospora*. Det var varierande resultat om de hade förekommit i kaffe någon gång innan eller inte samt om de ansågs vara potentiellt patogena. Det släkte som de flesta prover identifierades som tillhörde *Bacillus*, medans några släkten identifierades i ett prov.

Nyckelord: Mikrobiell tillväxt, Kallbryggt kaffe, Kaffekvarn

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1. Introduction

In the introduction part the problem description will be presented. The aim for this report will also be presented from the subjects the company in question wanted to get more knowledge about.

1.1. Problem description

Today the coffee grinders are not cleaned every day, because it is hard to reach every part and clean them in a good way. They are used until the grinding plates stop grinding the coffee beans in the wanted way, when the grinding plates are switched the grinder can be cleaned in an easier way. Cold brewed coffee beverage has become a popular coffee drink and is not heated in the process. The cold brewed coffee can therefore be contaminated from the coffee grinder due to build-up of coffee ground. The coffee drink is also more popular in warmer regions and therefore storage in warmer conditions may be more common, the warmer storage conditions may contribute to microbial growth in the coffee.

3TEMP ltd is a company located in Arvika, Sweden. They deliver and produce high-end coffee machines all over the world and they offer a wide range of coffee machines for all kind of business. Now they want to broaden their knowledge about hygiene of their products. They wanted to see if there is any microbial growth in the coffee grinders over time and how they could be cleaned in a good way to minimize the risk of microbial contaminated coffee. The problem also includes that they want to secure that the cold brew coffee drink is safe as well since it is not heated and therefore microbial contamination can occur in the drink. The company also wanted to be sure for how long cold brewed coffee could be stored, both in refrigerator and at room temperature. In this paper the microbial growth in coffee grinder and cold brewed coffee will be assessed and the cleaning process of the coffee grinder will be left out.

The fast brewing process of cold brewed coffee with the machines produced by the company makes that the company is leading at cold brewed coffee, therefore it is of importance to know what the best storage conditions for the ready-made coffee drink is. The company therefore wanted to know more about the microbial growth in cold brewed coffee, when stored in refrigerator and at room temperature, they

were also interested in the microbial growth and the differences if stored open or closed and whether the growth increased during storage.

Earlier research about cold brewed coffee has not been made, but the database used was Google Scholar and the main search phrases were: *Microbial growth in coffee*, *Bacterial growth in coffee*, *Mould growth in coffee* and *Coffee microbial growth* and in this way most of earlier research about coffee beans could be used in this project as something to base the findings on.

1.2. Aims and purpose

The aims of this paper are to investigate the microbial growth in a coffee grinder that have been used for a longer period of time and have not been cleaned beforehand and in this way make sure that the conditions during the experiment is the same as in reality. The aim also includes the investigation about the microbial growth in cold brewed coffee drink at 4°C, refrigerator, and 25°C as well if the glass flasks are stored open or closed and up to 5 days of storage.

The reason for storage up to 5 days is to be able to see if the microbial growth increases or not over time and 5 days should be enough for the microorganisms present to grow and another reason is that the coffee usually is stored for no more than 5 days.

The paper will also describe the different microorganisms found and whether those have been found in coffee in earlier studies.

Research questions:

Is there any microbial growth in a coffee grinder?

Is there any microbial growth in cold brewed coffee beverage?

Under which storing conditions does most of the growth occur?

2. Background

The background part will focus on the history of coffee, the consumption of coffee, production of coffee from fruit to finished product, the factors that will affect the quality of coffee and what quality parameters that are important followed by the milling process of coffee beans. Then proceeding with how our health may be affected by coffee. After those more introducing parts to coffee it will continue with defining what cold brewed coffee beverage is and the brewing process. At last the microbial growth in coffee beans will be assessed.

2.1. History of coffee

Coffee grown worldwide can trace its heritage back for centuries, (Ncausa, 2020b), to the coffee forests of Ethiopia and when brought east to Arabia coffee started its journey across the world.

The habit of drinking coffee is said to first have emerged in Arabia, (Teketay, 1999), about in the middle of the fifteenth century, but this was thought to be more of liquor. The art of roasting and preparing a drink from the grounded beans was thought to have started in Persia. From here the habit of drinking coffee did spread all over the world, reaching Europe in early 1600s and across the Atlantic Ocean in late 1600s. The first time that coffee was said to be drunk in England, (Wellman, 1961), was about 1651 and from there the drink was much appreciated and a business of import was built up. From England it spread to France where the popularity of the drink grew fast and coffee houses expanded quickly.

When coffee came to the New world, (Ncausa, 2020b), or New York to start with, it grew in popularity. But tea was still the most liked beverage of the two until 1773 when colonists revolt against the heavy tax that was put on tea, thus resulting in The Boston Tea Party, and from this point the American preference against coffee changed.

2.2. Consumption and production of coffee in the world

One of the reasons to a growing consumption, (Holscher & Steinhart, 1995), of coffee is the flavour of the finished product.

Coffee is the most consumed beverage after water, (Butt & Sultan, 2011), and people from all over the world are consuming about 500 billion cups of coffee annually. Coffee is also the second largest trading commodity after oil (Kingston, 2015). It is 70 countries, (Butt & Sultan, 2011), that cultivate coffee, but Brazil, Colombia, Ethiopia and India are the main producers.

The coffee beans that are used for coffee production are from two, (Nilsson, 2009), different species. Namely Arabica, *Coffea arabica*, and Robusta, *Coffea canephora* var. *robusta.* Today coffee is cultivated in many countries, and each country can provide different qualities, such as different aroma and flavour.

2.3. Production of coffee – from fruit to finished product

In this section the way of production will be assessed, which steps that is included in the production of coffee from the harvest until finished product in a package.

The way of the production of coffee usually has the same order independently on the kind of coffee (Nilsson, 2016).

2.3.1. Harvest

The first step is the harvest of the coffee fruits (Nilsson, 2016). The fruits mature at different points and the unripe, ripe and over-ripe fruits grows on the tree next to each other.

The harvest occurs in different times of the year (Nilsson, 2016) depending where in the world the coffee grows. North of the equator the harvest is between September and December meanwhile south of the equator the harvest occurs between April and August.

Coffee beans can be harvested both by hand and by machines (Nilsson, 2016), the most common way is still to harvest by hand. Hand harvest with care is a crucial point in producing coffee of high quality since only the ripen fruits will be picked. The harvest by hand can also be faster by harvesting all fruits at once, but this require sorting afterwards since fruits that also are unripen and over-ripen will be harvested. Harvesting with a machine usually occur in Brazil and on places where the coffee plants are located on flatter surfaces. All fruits are harvested at once and the crucial part is therefore the sorting of the fruits afterwards.

2.3.2. Processing methods

There are two different processing methods for coffee, (Selmar *et al.*, 2006), wet processing and dry processing.

In wet processing the pulp in the coffee cherries is removed mechanically and the mucilaginous residues from the pulp are degraded by fermentation (Selmar *et al.*, 2006), the other part of the coffee is dried and hulled. On the contrary the dry processing, the entire coffee fruits are dried including the pulp and are husked directly.

After the processing the coffee beans are collected in bags and stored for 1-2 months (Nilsson, 2016), it is mentioned that if the coffee is not allowed to rest it will have a disagreeable taste.

2.3.3. Shipping and sorting

After the processing, and the rest in bags, the protecting layer of the coffee beans are removed (Nilsson, 2016). The protecting layers are removed before the shipping to the buyer to lower the volume and weight of the coffee and thus also lowering the price for transportation. Directly after the removing of protecting layers the beans are sorted after size and quality properties, beans that still have the protecting layers, discolouration or not fully developed are removed either by machines or by hand.

There is always a test sample and tasting at the roasters in advance (Nilsson, 2016). The roasters make sure that everything is as expected with the coffee beans and make light roasted coffee to be able to identify any inaccuracies with the beans. The tasting is preformed both by smell and by taste. The taste is only a sip of coffee because the first experience is the most important aspect for the consumer.

The packaging of the coffee for transport to the buyer is different (Nilsson, 2016) depending on the size of the roasters or if it is a special coffee. The size of the packages is either in large containers covered in plastic on the inside or in bags made out of jute. If it is a special coffee the bags are covered in a protecting material that can withstand the moist better and the very nice special coffees are packaged in vacuum bags. When the coffee arrives to the roasters a test sample and testing is repeated to make sure that the coffees are the same and have the same quality.

2.3.4. Blending and roasting

The last two steps before the coffee is ready to be grinded are the blending of different coffee beans and the roasting process. The blending can be both before and after the roasting. The blending of coffee beans is important for big roasters since the taste of their coffee have to be the same all the time and this can be achieved by using different sorts of beans and also different amount of different beans. One blend of coffee can consist of 5 to 10 different sorts of coffee beans.

The roasting step is the main step in the processing chain of coffee (Nilsson, 2016). The roasting changes the characteristics, structure and appearance radically of the beans. Almost all water in the beans evaporates and the compounds that give coffee its taste and aroma are released. The aroma comes from essential oils that are located in the cells of the coffee beans.

There are different roasting degrees (Nilsson, 2016), such as light roast and dark roast; the difference is the temperature of the roaster. Light roast beans are heated up to 170-180°C and dark roast beans are heated up to 200-205°C. It is important that the beans are roasted to perfection because if they are roasted too little all the oils will not give flavour and aroma and this will make the coffee weak and uninteresting, meanwhile if the beans are roasted for too long the oils will evaporate and this will make the coffee bitter and the body vanishes.

The flavour compounds are mainly formed during roasting (Holscher & Steinhart, 1995), but it is over 200 known compounds in the green coffee beans and in the roasted coffee beans about 800 different volatile compounds have been found.

2.4. Factors affecting quality and quality parameters

In this section the quality parameters that are important will be assessed, those can affect for example the aroma and taste of the coffee. The definition of quality from different perspectives will also be mentioned.

2.4.1. Quality parameters

The moisture content is an important indicator (Leroy *et al.*, 2006) for quality and it is the most important defect for the trader and the roaster. High moisture content is a loss of material and will cause physical and sensorial defects, when the beans have moisture content over 12.5% the risk of moulds forming during storage increases, but with moisture content lower than 8% the beans will lose flavour.

The physical appearance is also a quality parameter (Leroy *et al.*, 2006) that is important and it is mainly referring to if the beans are damaged in some way as well as the size of the beans. The size is important because small beans of same variety can be lower in price. It is also important that the beans are the same size when roasting. This is important due to that if some beans are smaller, they might end up to be burnt and larger beans might be under-roasted, the differences in size will affect the appearance of the beans and most importantly the cup quality.

When coffee beans are harvested it must be done carefully (Teketay, 1999), since this will affect aroma, taste and other qualities of the product. The fruit also has to be harvested when ripe, since un-ripe and overripe fruits will give a poorer grade coffee. It is agreed that if the coffee beans are hand-picked this will produce the

best quality green coffee (Leroy *et al.*, 2006), because the defects in the coffee batches will be lower than when the beans are picked with a machine.

The different methods of drying the coffee beans affect the aroma (Teketay, 1999) in different ways. In for example Ethiopia the coffee is dried on the ground and this creates an earthy and muddy taste. During the drying variations in the structure of the beans (Coradi *et al.*, 2007), such as colour, defects and flavour, can affect the quality of the beverage. The quality potential can be reduced also by external factors. External factors are for example temperature, relative humidity and mechanical damage, for example the structure of the membranes could have been change. To obtain good quality in the product the best drying temperature is around 40°C, but to be able to maintain the quality of the coffee it is not enough with good drying but the storing is also important. Dry processing is avoided for quality samples (Leroy *et al.*, 2006), since this will enhance the bitterness.

For the consumer the main quality aspect is about the health (Leroy *et al.*, 2006). Coffee contains many molecules that can be connected to health and alertness. Some molecules exist naturally in the coffee, they can be derived from biochemical reactions after the roasting. The molecules can also be external compounds that do not have anything to do with the chemical composition of the coffee beans. Example of external compounds are ochratoxin A and residues of pesticides.

2.4.2. Definition of quality

The definition of quality for coffee has probably evolved over the years (Leroy *et al.*, 2006). The definition varies depending on where in the production-consumer line it is mentioned.

Quality for the farmer is a combination of amount produced (Leroy *et al.*, 2006), the price and how easy it is to cultivate.

For exporter and importer the quality might be the size of the beans (Leroy *et al.*, 2006), lack of defects, the physical appearance and the price.

For the roaster quality of coffee is dependent on moisture content (Leroy *et al.*, 2006), how stable the appearances of the beans are, origin, the composition of biochemical compounds and organoleptic quality. The organoleptic quality can be defined by each consumer market and country so this may be different. This aspect differs usually depending on nationality and costumers' expectations.

When quality is mentioned by the consumer it is usually referring to the price (Leroy *et al.*, 2006), taste and flavour, the effects on health and alertness, geographical origin and also the environmental and sociological aspects, this can be if the coffee is organic or fair trade etcetera.

2.5. Coffee grinders and the milling process

The coffee grinder is one of the crucial tools when coffee is prepared (Kingston, 2015). If the grinder is of low quality it can result in coffee with low quality in the end. There are two types of coffee grinders, one with knives and one with milling plates. Milling plates are superior to the knives, because the milling plates will grind the coffee nicely and, in that way, release more aroma from the coffee beans.

With milling plates the coffee may have a more bitter taste (Kingston, 2015), because it will make more contact with the water when brewed. The grinders with milling plates can grind coffee for all types of coffee, depending on what type of coffee that is made the finer or harsher the coffee will be grinded. The distance between the milling plates can be controlled, this is especially important for espresso coffee.

The grinders with knives are most controlled by the time of grinding (Kingston, 2015) and therefore it is important to know how every milling type of coffee looks like. The knives cut the beans instead of crushing them and this result in an uneven result and the extraction in water will have lower quality.

The temperature is also one important aspect (Kingston, 2015), since if the beans are heated enough oils and aromas can be released, but this is not very common to occur. Coffee beans should always be newly grinded, since the aromas and the oils will be vaporized in 15 minutes.

The method and how finely grinded the coffee is (Kingston, 2015), are of great importance for the caffeine content in the finished drink. The smaller the beans are grinded the more caffeine will be extracted from the beans. By the choice of how fine the beans are grinded the optimal taste can be obtained for each and every brewing method. Cold brewed coffee is the brewing method where the grains are the biggest and Turkish coffee will use the finest grinded coffee.

2.6. Human health affected by coffee and caffeine

In this part the affect coffee and caffeine have on human health will be assessed, both the positive and negative effects. There will also be a little bit about the environmental impact that coffee and caffeine have.

2.6.1. Coffee

Coffee have a multitude of constituents (Preda *et al.*, 2019), where some have actions that are known and others that are unknown. In some studies, there have been claims that coffee could have a protective effect towards some diseases such as diabetes type 2 and Parkinson´s disease, but at the same time there are also studies

that warn about possible adverse effects of coffee. Coffee could have effect on our gut microbiota, there could be changes triggered by the effect of caffeine. Effects of caffeine have proven to be, for example, promoting of gastroesophageal reflux, stimulation of gallbladder contraction and colonic motor activity.

There are claims about the health promoting potential that coffee has (Butt & Sultan, 2011). Those health-promoting properties are often correlated with the richness of phytochemicals that for example includes caffeine. Coffee can provide protection against cardiovascular disease, diabetes mellitus, Parkinson´s disease, Alzheimer´s disease, DNA damage and also improve antioxidant status of the body. Coffee consumption has also been negatively correlated with the metabolic syndrome, because the components acts as antioxidants and for example intake can decrease insulin sensitivity. Coffee has shown to correlate to multiple potential health benefits (Rao & Fuller, 2018), a reduction in health risks has been found when three cups of coffee was consumed. The consumption of coffee can be associated with a decreased risk of liver-, metabolic- and neurological diseases. This can be because coffee has a high antioxidant level as well as anti-inflammatory effect.

Coffee is also contributing to alertness through stimulating functions. On the contrary high consumption will cause high consumption of caffeine (Butt & Sultan, 2011).

2.6.2. Caffeine

Caffeine is a natural alkaloid with anti-herbivorous properties (Vilanova *et al.*, 2015) and is produced by *Coffea arabica* and *Coffea canephora*. Caffeine is a well-known bioactive compound that have stimulating effects on the central nervous system, as well as other potentially positive effects on human health. Positive effects on human health is though linked to living styles and it is therefore difficult to draw epidemiological conclusions linking caffeine to health.

Caffeine is also addictive (Butt & Sultan, 2011) and when not consumed can lead to headache and muscle pain. Caffeine can also be a risk factor for breast cancer and prostate cancer, but also an increased risk of osteoporosis with consumption increase of coffee.

Caffeine have been proposed both as an environmental pollutant and also as an easily detectable marker for untreated wastewater, and this can be strengthened by the fact when caffeine is present in natural environment it is one of the best indicators of anthropogenic contamination. The effects of caffeine on human health and on the environment has therefore led to that the decaffeination process was developed. It is now under proposition that the decaffeination process could be made with microorganisms (Vilanova *et al.*, 2015).

2.7. Cold brewed coffee and the brewing procedure

In this section a description about cold brewed coffee is presented and also how the brewing procedure is done when it comes to cold brewed coffee, usually and the differences from the machine used at 3TEMP in this project.

2.7.1. Explanation of cold brewed coffee

Cold brew coffee is made of grounded and roasted coffee beans (Ncausa, 2020a). The roasted coffee is extracted in cooler temperatures. The brewing process takes longer time, therefore trades time for temperature to be able to extract optimal flavour profile from the beans.

Cold brew coffee is also a type of brewing method (Ncausa, 2020a) and not a type of serving method. Cold brew coffee is only cold brew if the coffee has been extracted in cooler temperatures.

2.7.2. Brewing procedure

Usually for cold brewed coffee it is brewed for a long time (Kingston, 2015), sometimes up to 24 hours and this is made with cold water, about 20-25°C. The result will be a beverage that has much body, which means that the coffee is dark and strong in taste. The most common way is to put coarse grinded coffee in cold water for about 12 hours and afterwards the coffee is run through a filter.

Cold brew coffee has a low acid content (Kingston, 2015) since some oils and fatty acids only is released at higher temperatures. This can cause that those who have a taste for espresso might think that cold brewed coffee is tasteless, because oils released at higher temperatures have a strong impact on the taste profile.

In this project a machine from 3TEMP was used and instead of a brewing time up to 24 hours the machine will brew cold brewed coffee within 20 minutes and thereby reducing the time but still get the same taste as if would have been brewed for up to 24 hours. It focuses more on the flowrate, temperature and extraction time as the three critical points (3TEMP, 2020).

2.8. Microbial growth in coffee beans

The microbial growth section will assess the microorganisms that grow on and inside the fruit and then try to examine the roasted coffee beans and also cover earlier research regarding microorganisms found in roasted coffee beans

2.8.1. Microbial growth on the surface and inside the coffee fruits

The growth of moulds on the coffee bean can begin in the field at the harvest stage (Clarke & Macrae, 1988) but also on the coffee tree (Bucheli & Taniwaki, 2002), during the ripening process of coffee cherry or take place within the developing coffee bean during cherry maturation.

It can happen, especially in dry processing (Clarke & Macrae, 1988), that the coffee beans have to be stored in transport sacks before they can be spread on the drying area. When the coffee beans are leaved in sacks, they will start to heat up due to prolific growth of bacteria and moulds. When heating up the temperature can reach 40-50°C causing thermophilic bacteria and moulds to reproduce, known thermophilic bacteria *Lactobacillus delbrueckii* and *L. leichmannii* and the mould *Aspergillus fumigatus*. Since bacteria require higher water activity than mould it is likely that the bacteria will stop growing after a successful drying period. The general statement concerning process time is that it should be as short as possible to be able to prevent formation of mycotoxins.

A study showed that the main mould flora on the surface of green coffee beans (Clarke & Macrae, 1988) was seven species of *Aspergillus* and the one that was found most frequently was *Aspergillus niger.* The same study concluded that toxin-producing food-borne mould genera such as *Penicillium, Fusarium* and *Alternaria* was present but in significantly lower level inside the coffee beans, than *Aspergillus*. But it was still considered that the mycotoxins connected with those will play an important role.

On the outside of green coffee beans many moulds from the genera *Aspergillus, Penicillium, Alternaria* and *Cladosporium* have been detected (Clarke & Macrae, 1988), those can produce mycotoxins. Most of the toxins were partially degraded during the roasting of the beans; this is especially regarding Aflatoxin B_1, Ochratoxin A and Sterigmatocystin. The levels of those toxins encountered have been quiet low and therefore the level present after roasting are considered to not be a risk for human health.

Later on with improved detection method it could be determined that ochratoxin A also occur in roasted coffee samples (Bucheli & Taniwaki, 2002) and the mould that often have been proposed for production of ochratoxin A in green coffee beans is *Aspergillus ochraceus* but the origin of ochratoxin A in coffee beans are still uncertain. Findings from several authors has led to the conclusion that mainly three aspergilli (Bucheli & Taniwaki, 2002); (Leroy *et al.*, 2006) are causing ochratoxin A production in coffee, those are *A. ochraceus, A. niger* and *A. carbonarius*. Those three aspergilli have different growth range (Bucheli & Taniwaki, 2002); *A. ochraceus* can grow in temperature of 8-37°C and a water activity of 0.95-0.99, *A. niger* can grow in temperature of 6-47°C and with a water activity of 0.77, germination occur and lastly *A. carbonarius* can grow in temperature of 8-41°C and water

activity of 0.90. The black spores of the black aspergilli give protection against sunlight and UV light and therefore the major habitat of those are on fruits that are dried in the sun without preservatives and this includes grapes, figs and coffee.

The transport (Clarke & Macrae, 1988), except the drying process, is another way the coffee beans could be spoiled. The spoilage could be because of wet weather conditions and prolonged transportation because they are tightly packed in sacks, this sort of spoilage is highly undesirable both for the roasters and the grower. Coffee beans that have longer transport in closed containers shows a higher incidence of secondary mould metabolites, such as mycotoxins, than coffee beans with shorter transportation in open or ventilated storage.

Coffee is a so called hygroscopic material (Magan & Aldred, 2005), which means that it can absorb water during transport and storing and this can cause the moisture content to be over 12-13% and this can cause *A. ochraceus* to grow and also the production of ochratoxin A is a risk in this case, if the conditions are the right. The optimal conditions for ochratoxin A production (Sumpavapol *et al.*) are 20°C and water activity is 0.99.

2.8.2. Microbial growth in roasted coffee beans

There are limiting factors of the coffee productions (Mengistu, 2008), those factors include species of fungi, bacteria, viruses, nematodes and insects. There are three major fungal disease and two major bacterial diseases that affect the production of coffee and it has been reports regarding that viruses also can affect the production amount of coffee in a negative way.

There have been reports stating that coffee has antimicrobial effects (Runti *et al.*, 2015), but the components that are responsible for those activities have not been fully elucidated yet. Though it has been stated that the antimicrobial effect is related to the roasting procedure of the coffee beans and more specifically the degree of roasting and therefore the Maillard reaction can be responsible for the antimicrobial effect.

2.8.3. Earlier reports regarding microbial growth in coffee

There has been evidence of presence of bacteria from the genera of *Acinetobacter, Bacillus* and some enterobacteria (Vilanova *et al.*, 2015) have been present during natural fermentation of coffee beans, at the same time *Paenibacillus* and have been proved to be abundant in composting process of coffee hulls.

A study made on Nespresso machines where the waste coffee containers were analysed showed that a varied bacterial community had grown in in the waste coffee containers (Vilanova *et al.*, 2015), but it was mainly for the evidence of which bacteria that could be used in the decaffeination process. In the study made the main

taxa that was detected belonged to *Enterococcus* and *Pseudomonas*, and other frequent genera was *Stenotrophomonas, Sphingobacterium, Acinetobacter* and to a lesser extent *Coprococcus*, *Paenibacillus* and *Agrobacterium*.

The study mentioned did however, for the first time, showed that coffee leach from capsule machines serves as a rich substrate for bacterial growth (Vilanova *et al.*, 2015), but also that the caffeine content does not prevent a rich bacterial diversity from colonising coffee leach rapidly.

3. Material and Methods

In this part the material and methods used will be explained. It will start with go through how the grinding and brewing of the cold brewed coffee was done and then continue with the different methods used. For each method there will also be established what was used and how the laboratory work was done. There will also be an explanation to why each method was used and what kind of result that was obtained for this project.

3.1. Grinding and brewing

The grinding and brewing took place at 3TEMP in Arvika. For the grinding a Ditting swiss grinder 804 was used with the size of the coffee grind was for Press coffee according to the machine used. The beans used were from Löfbergs coffee roastery in Karlstad, Sweden, and was Espresso beans. The bag had been standing open for recreating environment that coffee beans could be stored in at coffee shops before usage. The grinder had not been cleaned beforehand since this is not a process that is done every time the coffee is grinded. The grinders are not cleaned every time because hard to reach areas and therefore cleaning of the grinder occur when the grinding plates need to be switched.

The brewing process was done with one of the machines that the company is producing and therefore an already made program for cold brew was used. The brewing process took about 20 minutes to be done. After the brewing process the coffee was poured into autoclaved bottles and put in a cooling box for transport. The transport was about 4 hours between the company, 3TEMP ltd in Arvika and the university, SLU Ultuna in Uppsala. When at the university the bottles were marked accordingly to test protocol, see Table 1, and then put in the right conditions for incubation. The bottles were stored either at 4°C or 25°C and stored either open or closed in respective temperature. The ones stored at 25°C was incubated for 1, 3 and 5 days and the ones stored at 4°C was incubated for 3, 4 and 5 days. The bottles were stored open and closed and in different temperatures because this was one aspect that was asked from the company. This was requested because sometimes cold brewed coffee could be stored both open and closed and either in refrigerator or at room temperatures.

Table 1. Storing conditions for bottles with cold brewed coffee. Temperatures (4°C or 25°C), days of incubation (1, 3 and 5 at 25°C, 3, 4 and 5 at 4°C) and if bottles were open or closed

Temperature	Days of incubation	Open or closed
25°C	1	Open
25°C	1	Closed
4°C	3	Open
4°C	3	Closed
25°C	3	Open
25°C	3	Closed
4°C	4	Open
4°C	4	Closed
4°C	5	Open
4°C	5	Closed
25°C	5	Open
25°C	5	Closed

3.2. Swabbing of coffee grinder

The swabs used were from Avantor sciences and were of the type of transport swab that all were individually packed. The shaft was plastic and the head were viscose and the transport media was Stuart liquid.

The swabbing was tested on four different places on the coffee grinder at the company. The places that were swabbed were the milling plates, the splash zone behind the tube where the coffee comes out, the inside of the tube where the coffee comes out and the hand protection over the milling plates. For a visual showing of the places see Figure 1-4, Appendix 2, respectively for the places where the samples were collected.

After swabbing the swabs were put in a cooling box for transport from the company to the university, where the samples were plated and incubated accordingly, see the section Agar plates.

3.3. Agar plates

Agar plates used in this study was MEAC (Malt Extract Agar with Chloramphenicol) from Merck, TGEA (Tryptone Glucose Extract Agar) from Sigma aldrich and NA (Nutrient Agar) obtained from Albina Bakeeva. The MEAC and TGEA was prepared in the study and NA was already made and therefore those were used.

MEAC plates was prepared all at once and stored in 2°C until usage. MEAC media was prepared with correct amount of Malt Extract Agar with chloramphenicol (100 mg/l) and then autoclaved for 15 minutes at 121°C. Then left to cool down and then the plates were poured, using about 20mL for each petri dish and then left overnight to solidify. Afterwards plates were stored in 2°C until usage. The MEAC media was used for isolation of yeasts and later on moulds.

TGEA plates was made throughout the laboratory work, since for those plates pour-plating technique was applied. Different amount was therefore prepared different days depending on how many plates that were needed that day, see Table 2 for amount of TGEA plates used each day. In Table 2 the amount of MEAC plates used each time is also presented.

Table 2: Amount of TGEA and MEAC used each time

Days after incubation of bottles	Amount of TGEA plates	Amount of MEAC plates
1	6	6
3	12	12
4	6	6
5	12	12
Samples from Coffee grinder	12	12

When agar media had been weighed accordingly together with correct amount of cycloheximide (500 mg/l), deionized water was added in correct amount and thereafter the media was heated in microwave until boiling point, this varied depending on the amount but usually around 4-6 minutes. At the boiling point the liquid was clear and no clumps of agar was left. The agar was left to cool until it did not get warm to put it against the wrist. First layer of the agar was mixed with either the cold brewed coffee or the liquid from the swabs, see the section Plate spreading for further information, and then left to solidify, thereafter the second layer was poured. TGEA media was used for isolation of general aerobic bacteria. The MEAC plate was used as a general isolation media for yeast and moulds.

3.4. Plate spreading

MEAC and TGEA plates was spread the same days, see Table 2, for amount of plates used each day. For cold brewed coffee 0.1mL of sample was applied to the MEAC plate and 1mL of sample was added in an empty petri dish and the first layer of TGEA agar was added, the plates, with TGEA, was then swirled in the shape of an 8 to evenly distribute the sample in the first layer of the agar, when this layer had solidify the second layer was applied.

The samples from the swab test was added almost in the same manner for both types of media. All liquid from the swab test was used for both types of media. The

difference was that for MEAC media the liquid was poured on top and spread out with the swab itself and for TGEA the liquid was added to an empty petri dish and the first layer of agar was added and the plates was swirled in the shape of an 8, when the first layer had solidify it was stroked with the swab to enable more sample to stick to the agar plate and thus enable more bacterial growth afterwards the second layer was poured.

MEAC plates was incubated first upside down for 2 days to check for any yeast growth and then turned around and incubated for another 3 days to enable mould growth, they were incubated in 25°C. TGEA was incubated in 30°C and upside down for 3 days.

For NA one colony, or small amount of sample, from plates with growth was obtained and spread on either ¼ or ⅓ of the plate. NA was used to be able to run a PCR later on and for some of the samples also MALDI-TOF. NA was only used for bacteria and not for mould. When purifying mould for PCR, MEAC plates was used because of a lot of spores that had begun to form.

3.5. MALDI-TOF

MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight, is one of the most powerful tools for analysis of biomolecules (Jurinke *et al.*, 2004). MALDI-TOF was performed at SLU in the VHC building. The samples were collected and spread on the empty spots on the reading disc thereafter 1µl of matrix was added and set to dry completely before reading of the spots. The matrix contained Bruker HCCA portioned, water, acetonitrile and TFA.

MALDI-TOF was made in the laboratory work to complement the PCR and thus gaining more result.

MALDI-TOF has a clear advantage in the identification of bacteria (Lay Jr, 2001) and that is the speed of analysis and it can be considered to be complementary to data obtained from other methods for bacterial identification. MALDI-TOF is therefore mainly an identification method used in this project to mainly complement the findings from sequencing of bacteria run in PCR.

3.6. PCR

PCR (Polymerase Chain Reaction) was performed both for bacteria and moulds. The PCR was made to amplify the 16S region of bacteria and the D1/D2 region of the large ribosomal unit of mould. The steps for PCR for bacteria and moulds differ because you have to break down the cell wall of the moulds before they can be run in PCR. For bacteria colonies was picked up by a wood toothpick and then blended into 100µl of sterile pure water. Afterwards the master mix was prepared, the master

mix consisted of: 1µl forward primer 16Ss, 1µl reverse primer 16Sr, 12µl sterile Milli-Q water and 12µl DreamTaq green 2x Master Mix (contain buffer, Taq, dNTPs and loading dye for gel running later). The master mix was prepared accordingly how many samples that were being analysed and one extra to be able to have a negative control. After the master mix was prepared 24µl of it was added to each PCR tube. In the PCR tubes 2µl of the bacterial suspension was added. Then the PCR was run according to the PCR program for bacteria that is as follows (this was also pre-set in the PCR machine):

1. Initial denaturation: 95°C for 5 minutes
2. Denaturation: 94°C for 30 seconds
3. Annealing: 50-51°C for 30 seconds
4. Extension: 72°C for 2 minutes

Denaturation, annealing and extension is repeated 30 times

5. Final extension: 72°C for 10 minutes

Afterwards the PCR machine will hold a temperature of 16°C. Meanwhile PCR machine was running the gel was casted. The gel was made by first preparing 1% agarose in 0,5xTBE and then the appropriate tray-and comb size was chosen. 20µl of gel red was pipetted into a plastic mug and then it was filled to about ¼ with the agarose. The agarose was mixed and the gel was casted and let to solidify for about 15 minutes. The gel was transported to an electrophoresis tray with the same kind of buffer (0.5xTBE). the gel was then loaded first with 3µl of size marker and then 3µl of each sample from the PCR tubes. The gel was run 100V, 80 mA for about 30 minutes.

The procedure for moulds differs from bacteria in the way the samples need to be prepared, as mentioned for bacteria the sample could be obtained directly from the Nutrient Agar Plates, but for moulds it is a little bit different.

For mould preparation the moulds were first grown in Eppendorf tubes with 500µl glucose yeast medium for 2 days at 25°C on a shaking table. The samples were centrifuged at 13000 rpm, using a Clinical Bench Centrifuge from Allegra, for 5 minutes and the supernatant was pipetted and discarded. The moulds were then washed with 500µl TE buffer and then centrifuged again at 13000 rpm for 5 minutes. Again, the supernatant was pipetted and discarded. 300µl of extraction buffer was added and the solution was grinded with sterile pestles for a few minutes. 150µl 3M Na acetate of pH 5.2 was added and the tubes were placed at -20°C for 10 minutes. The samples were centrifuged at 13000 rpm for 5 minutes and the supernatant was transferred to a new tube and an equal amount of cold isopropanol

was added and then the tubes were placed in -20°C for about 15 minutes. The samples were then again centrifuged at 13000 rpm for 5 minutes and the supernatant was discarded. The samples were washed with 500µl of cold ethanol and again centrifuged at 13000 rpm for 5 minutes and the supernatant was discarded and the samples was centrifuged one last time at 13000 rpm for 5 minutes and then all liquid was removed from the samples. The samples were then resuspended in 50µl Tris for 10 minutes at 50°C. After this procedure it followed the same as for bacteria except that the primers used were different, the primers were ITS4 and ITS1, and the PCR program was a little different for moulds:

1. Initial denaturation: 94°C for 2 minutes
2. Denaturation: 94°C for 20-30 seconds
3. Annealing: 50°C for 20-30 seconds
4. Extension: 72°C for 1 minutes

Denaturation, annealing and extension is repeated 35 times

5. Final extension: 72°C for 10 minutes

After the PCR they followed the same procedure once again.

The gel electrophoresis was carried out to identify any signs of DNA and also to check that the negative control did not show any signs of DNA. In this way it could be stated that the samples could be send to Macrogen for identification. For Macrogen identification all samples were transferred from PCR tubes to Eppendorf tubes and then registered at Macrogen and then sent away for sequencing.

The PCR and gel electrophoresis did not gain any direct result, they were made to enable identification from sequences obtained from Macrogen.

4. Result

The result part will first go through which kind of microorganisms that were identified and with what technique and also during which growing conditions. After the presentation about which microorganisms that were identified those will be examined to get a better understanding about them and where they have been isolated from and also whether they cause diseases or not.

4.1. Identification of microorganisms

The initial spreading on TGEA and MEAC plates and the growth can be seen in Appendix 1. From the beginning triplets from each sample was plated on each media. From those plates samples was transferred to Nutrient Agar both for running MALDI-TOF for some samples but also for PCR later on. In Appendix 1 it could be seen on how many plates growth occurred in the first place and in which conditions. Growth of bacteria occurred on almost all the plates; it was no growth at all on the plates with swab samples from the splash zone of the coffee grinder. On some plates where growth occurred it had been more of a slimy consistency thus making the counting harder and for the two mould samples it had been a lot of spores forming on one of the plates that made that no counting could be made. Since the agar plates made was for enumeration of bacteria and mould it could not be stated how many of each bacterium that was growing on the plate or if it was a lot of different bacteria as well on one plate. What can be stated is that the numbers of bacteria did raise from day to day and that the growth was faster at 25°C than in 4°C, this can be stated because 1 mL of undiluted sample was plated and the number of bacteria was higher in the samples with 25°C than in 4°C and also the number of bacteria was higher in samples incubated for longer time.

MALDI-TOF was run on samples from cold brewed coffee that was stored for 1, 3 and 4 days in 4°C and 25°C. The results for the MALDI-TOF was varied between No Organism Identification Possible, Low-Confidence Identification and High-Confidence Identification. It was 12 samples that was run in MALDI-TOF and out of those it was seven samples that got the result No Organism Identification Possible, one that got Low-Confidence Identification and four that got High-Confidence Identification. The bacteria that was identified with high-confidence were

Staphylococcus hominis for two of the samples, *Acinetobacter lwoffii* and *Micrococcus luteus*. At this point all plates had not been incubated for the rightful amount of time and therefore those samples could not be run and even though there were colonies on the plates those did not get identified by the MALDI-TOF machine

PCR and gel electrophoresis were run on both samples from cold brewed coffee and swab samples from the coffee grinder. PCR was carried out on samples from the cold brewed coffee and from swab samples from the coffee grinder The PCR samples was sent to Macrogen for identification. It was 42 bacteria samples that was sent to Macrogen for identification and two mould samples, the files from Macrogen was run in BLAST to identify the bacteria and moulds. and of those four were not identified because of no significant similarity could be found, seven were not identified because the forward, reverse or both sequences were not good enough to run in BLAST and 31 could be identified, the mould samples were both identified, see Table 3-8 for the result from BLAST and if the results were either no significant similarity according to BLAST program, if strains were too blurry or not very clear to get a result from or which bacteria that was identified and from which sample the result was from. Table 3 illustrates what kind of bacteria were present in the cold brewed coffee stored at 25°C for 1 day. In Table 4 the bacteria present in cold brewed coffee stored at 4°C and 25°C for 3 days. Table 5 the identified bacteria from the cold brewed coffee stored at 4°C for 4 days is presented. Table 6 gives information about the identified bacteria from cold brewed coffee stored at 4°C and 25°C for 5 days. Table 7, that is the last table that will show identified bacteria, shows which bacteria that were identified from the coffee grinder and at last Table 8 shows the two identified mould samples from the experiment.

Table 3. Identified bacteria after 1 day of incubation at 25°C

From sample	Strains blurry No significant similarity Identified	Name of identified micro-organism
Coffee open, 25°C	Strains blurry	–
Coffee open, 25°C	Strains blurry	–
Coffee closed, 25°C	Identified	*Bacillus siamensis*
Coffee closed, 25°C	Identified	*Bacillus shackletonii*

In table 3 the samples had been stored at 25°C for 1 day. At this state there was two different bacteria identified, but two samples could not be identified due to blurry strains in the sequences obtained from Macrogen.

Table 4. Identified bacteria after 3 days of incubation at 4°C and 25°C

From sample	Strains blurry No significant similarity Identified	Name of identified micro-organism

Coffee open, 4°C	Identified	*Bacillus licheniformis*
Coffee open, 4°C	Identified	*Staphylococcus hominis*
Coffee open, 4°C	No significant similarity	–
Coffee open, 4°C	Identified	*Bacillus shackletonii*
Coffee open, 4°C	Identified	*Mycobacterium llatzerense*
Coffee closed, 4°C	Identified	*Bacillus shackletonii*
Coffee closed, 4°C	Identified	*Bacillus camelliae*
Coffee closed, 4°C	Identified	*Bacillus shackletonii*
Coffee closed, 4°C	Strains blurry	–
Coffee open, 25°C	Identified	*Staphylococcus warneri*
Coffee open, 25°C	Identified	*Bacillus cohnii*
Coffee open, 25°C	Identified	*Bacillus shackletonii*
Coffee closed, 25°C	Identified	*Bacillus shackletonii*
Coffee closed, 25°C	No significant similarity	–
Coffee closed, 25°C	Identified	*Staphylococcus hominis* subsp. *novobisepticus*

In Table 4 the identified bacteria from cold brewed coffee samples stored for 3 days at either 4°C or 25°Care specified. Not all samples could be identified, some had blurry strains from the beginning, but some samples did have strains that was not blurry but they could not be identified when run in BLAST.

Table 5. Identified bacteria after 4 days of incubation at 4°C

From sample	Strains blurry No significant similarity Identified	Name of identified micro-organism
Coffee open, 4°C	Identified	*Staphylococcus epidermidis*
Coffee open, 4°C	Identified	*Bacillus shackletonii*
Coffee open, 4°C	Identified	*Bacillus camelliae*
Coffee closed, 4°C	Identified	*Staphylococcus hominis*
Coffee closed, 4°C	Identified	*Paenibacillus cookie*

In Table 5 the identification of bacteria continues with the identified bacteria incubated for 4 days and at 4°C. In this case all of the samples sent to Macrogen could be identified by BLAST.

Table 6. Identified bacteria after 5 days of incubation at 4°C and 25°C

From sample	Strains blurry No significant similarity Identified	Name of identified micro-organism
Coffee open, 4°C	No significant similarity	–
Coffee open, 4°C	Identified	*Bacillus licheniformis*

Coffee closed, 4°C	Identified	*Kocuria rhizophila*
Coffee closed, 4°C	Identified	*Bacillus licheniformis*
Coffee closed, 4°C	Identified	*Bacillus licheniformis*
Coffee open, 25°C	No significant similarity	–
Coffee open, 25°C	Identified	*Bacillus licheniformis*
Coffee open, 25°C	Strains blurry	–
Coffee closed, 25°C	Identified	*Bacillus licheniformis*
Coffee closed, 25°C	Strains blurry	–

In Table 6 the identified bacteria after 5 days of incubation are presented. There were also samples that could not be identified due to blurry strains from the sequencing and also samples that did not have significant similarity and therefore could not be identified.

Table 7. Identified bacteria from swab test from coffee grinder

From sample	Strains blurry No significant similarity Identified	Name of identified micro-organism
Coffee grinder, milling plates	Strains blurry	–
Coffee grinder, milling plates	Identified	*Paenibacillus cookie*
Coffee grinder, inside tube	Identified	*Paenibacillus cookie*
Coffee grinder, inside tube	Identified	*Paenibacillus cookie*
Coffee grinder, inside tube	Identified	*Staphylococcus hominis*
Coffee grinder, inside tube	Identified	*Paenibacillus yonginensis*
Coffee grinder, hand protection	Identified	*Paenibacillus yonginensis*
Coffee grinder, hand protection	Strains blurry	–

In Table 7 the identified bacteria from the coffee grinder is presented. The samples from the coffee grinder was taken with swab test as explained earlier in the material and methods part. In this case there were two samples that had blurry strains and the rest of the samples was identified, thus from the beginning the swab test were taken from four different places but not all did show any growth.

Table 8. Identified moulds from the laboratory work

From sample	Strains blurry No significant similarity Identified	Name of identified micro-organism
Coffee grinder, milling plates	Identified	*Neurospora sitophila* (teleomorph) *Chrysonilia sitophila*
Coffee closed, 25°C, 5 days of incubation	Identified	*Bjerkandera adusta*

In Table 8 the two mould samples that were identified are presented. One of the samples was from the coffee grinder and the second sample was from the cold brewed coffee stored at 25°C for 5 days.

From this result it can be seen that the main genera of bacteria were *Bacillus* although there was not growth on each agar plate and some samples could not be identified due to either blurry strains or that significant similarity was missing, the most abundant genera would have been *Bacillus*. The bacterium that was detected most times was *Bacillus shackletonii* followed by *Bacillus licheniformis* and those were identified in 7 and 6 samples respectively.

In Appendix 1 there is also reported how many colonies that were present on each agar plate. Not all colonies were purified on Nutrient agar but only those that looked differently from each other and this could mean that not all microorganisms were identified. It can also be seen that the growth rate was lower in those containers that was stored at 4°C than at 25°C. The result also shows that the growth rate increased when the container was stored open instead of closed, even though this did not prevent growth and neither did the cooler storage temperature. In Appendix 1 the number of colonies are presented, for the TGEA media 1 mL of undiluted sample was used and therefore the number of colonies are the total number of aerobic bacteria per mL.

4.2. About the identified microorganisms

In this section the identified microorganisms will be assessed. They will be described how they look like and what kind of abilities they have. It will also be presented if they have been found in relation with coffee in earlier studies.

4.2.1. *Acinetobacter lwoffii*

The first *Acinetobacter* was identified in 1911 (Percival & Williams, 2014), but it was given the name *Micrococcus calcoaceticus* because it was isolated from soil. Since 1911 at least 15 ‘generic’ names have been used to describe *Acinetobacter*. It was proposed by French microbiologists that the genus *Acinetobacter* as one comprising of non-motile, gram-negative, oxidase-positive and oxidase-negative saprophytes distinguished from other bacteria by their lack of pigmentation when grown on agar media and first in 1971 it was stated that *Acinetobacter* only should contain the oxidase-negative strains.

Acinetobacter lwoffii is a non-fermentative (Regalado *et al.*, 2009); (Berlau *et al.*, 1999), aerobic Gram-negative bacillus that occur as a natural flora of the skin and oropharynx, the mucous membranes, in approximately 25% of healthy individuals. *Acinetobacter* are coccobacilli (Wanger *et al.*, 2017) and when Gram stained they can resemble *Neisseria* species. When grown on MacConkey they appear as

non-fermenters and do not produce oxidase. *Acinetobacter* are strictly aerobic (Percival & Williams, 2014), short and plump rod-shaped bacteria, they are often capsulated and non-motile. *Acinetobacter* are ubiquitous, which means that they can be found everywhere, free-living saprophytes, which means that they live of dead or decaying organic matter. *Acinetobacter* cells are typically 1-1.5µm in diameter and 1.5-2.5µm in length. *Acinetobacter* are non-motile bacteria and the origin of the name reflects this, stemming from the Greek word 'akinetos' meaning 'unable to move'. *Acinetobacter* are catalase-positive, indole-negative and oxidase-negative and often encapsulated. On media such as nutrient agar *Acinetobacter* form smooth, sometimes mucoid, pale yellow to greyish white colonies, about 1-2 mm in diameter.

The majority of *Acinetobacter* are not dependent on a single growth factor (Percival & Williams, 2014) and are able to exploit a large number of organic carbon and energy sources. Aliphatic alcohols, some amino acids, dicarboxylic and fatty acids, unbranched hydrocarbons and sugars can all be metabolized by *Acinetobacter*, but they are non-fermentative and most cannot reduce nitrate to nitrite but there have been reports that state that some strains use both nitrate and nitrite as nitrogen sources.

It has a ubiquitous nature (Regalado *et al.*, 2009) and can therefore be seen as a potential opportunistic pathogen in patients with impaired immune system. *A. lwoffii* has also been found to be the cause of nosocomial infections such as pneumonia, meningitis, urinary tract infections as well as skin and wound infections. They are also resistant against commonly used disinfectants, irradiation and desiccation (Rathinavelu *et al.*, 2003); (Regalado *et al.*, 2009). *A. lwoffii* has also been found to cause bacteremia (Wanger *et al.*, 2017) in hospitalized patients.

A. lwoffii is, due to its ubiquitous properties (Rathinavelu *et al.*, 2003), frequently found in soil, water and dry environments and also predominantly found in foods. *A. lwoffii* has been found in frozen foods (Rathinavelu *et al.*, 2003); (Regalado *et al.*, 2009) such as bacon and chicken, it has also been found in pasteurized milk, the explanation to that they have been found in those foods is that they can survive a broad temperature range, low pH and dry conditions. *Acinetobacter* can be present in drinking water (Percival & Williams, 2014) but it has not been shown that drinking water is a transmission pathway, it is more likely that *Acinetobacter* is spread from person to person and from medical equipment to person.

Acinetobacter lwoffii reported in coffee

There have been studies where *Acinetobacter* have been found in coffee vending machines (Choi *et al.*, 2012), though it was *A. schindleri* and it was not stated whether it was from the coffee itself or if it was obtained from the machine, it was just stated that it was isolated from coffee vending machines.

4.2.2. *Bacillus camelliae*

Bacillus camelliae is also known as *Bacillus* strain 7578-1 (Niu *et al.*, 2018) and considered a novel bacterial strain. The name comes from that the strain was isolated from fermented green tea, *Camellia sinensis*. Based on Niu *et.al* (2018) polyphasic taxonomic data they propose that the isolate 7578-1 should be classified as representing a novel *Bacillus* species and thus have the name *Bacillus camelliae*.

B. camelliae cells are Gram-positive (Niu *et al.*, 2018), aerobic, terminal ellipsoidal endospore forming in the slightly swollen sporangium, motile with peritrichous flagella and rod-shaped with 0.3-0.5 µm in width and 2.5-4.0 µm in length. Growth occur between 20°C and 55°C, with an optimum at 30-45°C. It is oxidase-positive and catalase-positive.

Bacillus camelliae reported in coffee

Bacillus camelliae has only been presented to be isolated from fermented green tea and this is the first time, known until today, it is isolated from coffee; therefore, it is hard to provide a deeper explanation about this bacterium and also impossible to state anything more about this specific bacterium.

4.2.3. *Bacillus cohnii*

The name *Bacillus cohnii* was first proposed in 1993 (Spanka & Fritze, 1993), when those strains included in *B. cohnii* was first considered to be strains of a new species. The strains were named after German botanist Ferdinand Cohn, who described the genus *Bacillus* in 1872.

Bacillus cohnii are obligate alkaliphilic (Tekin *et al.*, 2012), Gram-positive, motile and rod-shaped with terminal, ellipsoidal endospores. *B, cohnii* produces alkaline protease that have optimal activity at 50°C and pH 10.0, but it has activity in the range of 30 and 75°C. The alkaline protease is described as extremely stable. The cell width is 0.6-0.7 µm (Spanka & Fritze, 1993), on alkaline meat extract-peptone medium the colonies are cream white and after incubation in 45°C for 2 days the colonies are 1-2 mm in diameter. *B. cohnii* is catalase and oxidase positive and nitrate is reduced, the interval of growth is from 10°C up to 47°C. *B. cohnii* samples from Spanka and Fritze (1993) was isolated from soil. In the cell wall diaminopimelic acid is replaced by ornithine and aspartic acid forms the interpeptide bridge thus creating the alkaliphilic properties.

Alkaliphilic *Bacillus* strains constitute an important source of extracellular enzymes (Tekin *et al.*, 2012) that are useful in numerous industrial processes, those strains secrete large amounts of alkaline proteases with high stability at elevated pH and temperature values. Alkaliphilic *Bacillus* do not typically grow well (Logan & Vos, 2009) on routine media such as nutrient agar, due to that it can grow at higher pH.

Bacillus cohnii reported in coffee

For several years alkaline pectinases produced by alkaliphilic *Bacillus* have been used frequently in industrial and biotechnological processes (Li *et al.*, 2005), those processes include textile and plant fibre processing, fermentation of tea and coffee.

4.2.4. *Bacillus licheniformis*

Bacillus licheniformis is commonly found in soil (Salkinoja-Salonen *et al.*, 1999) and is one of three *Bacillus* spp. that is part of the *Bacillus subtilis* group.

B. licheniformis is rod-shaped (Veith *et al.*, 2004), Gram-positive bacterium. In the soil it tends to form spores and is therefore desirable to be used for industrial purposes. The optimal growth is at 50°C but it can survive much higher temperatures, optimal temperature for enzyme secretion is at 37°C. By turning into sporeform it can survive harsh temperatures and when conditions are good it will turn back to vegetative state.

Bacillus licheniformis have been implicating to be a cause of food poisoning (Logan & Vos, 2009) as well as other human and animal infections, the resistance of the spores against heat, radiation, disinfectants and desiccation results in that *Bacillus* species are troublesome in the operating room, on surgical dressings, in pharmaceutical products and in foods. *B. licheniformis* has as mentioned been implicating food poisoning but do not appear to produce any toxin (Thwaite & Atkins, 2012), although some strains do appear to produce bacitracin. The production of bacitracin by *B. licheniformis* is a function of the cells (Bernlohr & Novelli, 1960) in the stage after growth and before sporulation, but the antibiotic is released only under conditions that will support spore formation.

B. licheniformis is a saprophytic bacterium (Attachment I -- Final Risk Assessment of *Bacillus licheniformis*) that is widespread in nature and is thought to contribute substantially to nutrient cycling because of the diversity of enzymes that are produced by the different strains.

B. licheniformis do also have plant growth-promoting activity (Gutiérrez-Mañero *et al.*, 2001) due to that it produces auxin and/or gibberellins, it has been at least one study that showed stem elongation when *B. licheniformis* have been inoculated to the plant, and it also increased leaf area.

Bacillus species are known to have roles in the post-harvest processing (Logan, 2012) and flavour development of cocoa, coffee and vanilla and in the production of several traditional fermented foods based on leaves and seeds. It has become appreciated that aerobic endospore-formers growing in rhizosphere may promote plant growth by nitrogen fixation and some strains of *B. licheniformis*, along with other *Bacillus* bacteria, have been isolated from the inner tissues of healthy plants and seems to have important roles in growth promotion and plant protection.

B. licheniformis is common in food (Mikkola *et al.*, 2000) and is usually regarded harmless, it can grow both anaerobically and aerobically in foods, some

toxic strains have though been found in for example baby formula and milk products.

For the food industry it is especially the aerobic endospore-forming bacteria that have been a trouble (Heyndrickx, 2011), among those the *Bacillus* and related genera is included. There are several explanations to this phenomenon and most of them are in relation with the spore characteristics, such as their ubiquitous presence in soil, their resistance to heat in common industrial processes, for example pasteurization, and also the adhesive characters of some spores that facilitate their attachment to processing equipment. It seems that the food industry is confronted with tolerant or resistant spore-forming and this might be a side effect of the use of new ingredients as well as the application of new processing and packaging technologies.

Bacillus licheniformis reported in coffee

B. licheniformis have the ability to grow at a low pH (Sierra-Lopera *et al.*, 2017) and because of this ability it can be present in liquid coffee extract. Liquid coffee extract is when the aqueous extraction made from roasted coffee beans has been concentrated. Liquid coffee extract that is used as a base ingredient in cocktails and liquor, beverages in dispensing machines, cookies, pastries and ice cream presents a high risk of contamination by *B. licheniformis*.

4.2.5. *Bacillus shackletonii*

Bacillus shackletonii has been identified from mossy soil on Candlemas Island (Logan *et al.*, 2004b) and the name shackletonii was obtained from the ship Shackleton that was used on the first British expedition to Candlemas Island.

The cells of *B. shackletonii* are motile (Logan *et al.*, 2004b) with round-ended rods and they occur singly. It is considered to be Gram-variable, Gram-positive reactions are only seen in cultures at temperatures below 30°C. The endospores usually cause the sporangia to swell. Minimum temperature of growth is between 15 and 20°C, the optimum temperature of growth is at 35-40°C and the maximum temperature of growth is 50-55°C. They are strictly aerobic and catalase positive.

Bacillus shackletonii reported in coffee

It is the same with *B. shackletonii* as with *B. camelliae* that it had not been observed in many different studies and thus it is less information given about this bacterium as well and as far as known for now it is the first time it is observed in coffee. This was also one of the most identified bacteria in this study.

4.2.6. *Bacillus siamensis*

Bacillus siamensis was isolated in Thailand (Sumpavapol *et al.*, 2009) and got its name from the old name of Thailand, Siam.

The cells are Gram-positive (Sumpavapol *et al.*, 2009), facultatively anaerobic and rod-shaped, the cells can exist singly, in pairs or more seldom in short chains. They are motile with peritrichous flagella. It is catalase-positive and oxidase-negative. Growth occurs at 4-55°C, but the optimum growth temperature is 37°C. *B. siamensis* is a species with halophilic properties (Jeong *et al.*, 2012) and was isolated from a type of salted crab product eaten in Thailand. It has halophilic properties since it can grow in an environment with up to 14% of NaCl.

Facultative anaerobic means that the organism can survive in the presence of oxygen and use oxygen in aerobic respiration, but is also able to survive without oxygen via fermentation, anaerobic respiration (Biologydictionary, 2018)

During screening of *Bacillus* species that produce novel antibiotic compounds it was found that *Bacillus siamensis* inhibited mycelial growth (Jeong *et al.*, 2012) of the plant-pathogen fungi *Rhizoctonia solani* and *Botrytis cinereal*, it also exhibited strong antibacterial activity against the Gram-positive bacterium *Micrococcus luteus*.

Bacillus siamensis reported in coffee

Upon this time there could not been found any studies made about whether or not *B. siamensis* have been isolated from coffee and therefore no evidence could be provided that this have happened before or not. *B. siamensis* has also mainly been isolated from salted crab in Thailand.

4.2.7. *Kocuria rhizophila*

Kocuria rhizophila was first isolated from the rhizosphere of narrowleaf cattail (Takarada *et al.*, 2008).

K. rhizophila is a coccoid (Takarada *et al.*, 2008), Gram-positive bacterium and the type strain was isolated from the rhizosphere of narrowleaf cattail. The cells are spherical (Kovács *et al.*, 1999) and exists in pairs, tetrads and packets, they are 1.0-1.5µm diameter and non-motile, endospores are not produced. The colonies are 1.5-2.5mm in diameter, opaque, smooth with irregular edges that are yellowish. It is aerobic and no growth occur over 40°C. *K. rhizophila* is a halotolerant (Takarada *et al.*, 2008) species that tolerate up 10% NaCl in the growth medium.

K. rhizophila has potentially robust cell structures (Fujita *et al.*, 2006) against organic solvents, for example when exposed to lethal concentrations of alcohol it did not show any significantly changes in the fluidity of the cytoplasmic membrane.

Members of the genus *Kocuria* have been isolated from a variety of natural sources (Takarada *et al.*, 2008), some of them are mammalian skin, soil, the rhizosphere, fermented foods and freshwater. It is also important when it comes to an industrial viewpoint, a special strain of *K. rhizophila*, earlier *Micrococcus luteus*, is designated as a quality control strain in several applications, for example susceptibility assays for a variety of antibiotics, but despite of ecological and industrial importance no complete genome information is currently available for bacteria in *Kocuria*/*Micrococcus* group. In 2003 a strain of *Micrococcus luteus* was proposed to change from *Micrococcus* to *Kocuria* (Tang & Gillevet, 2003) and thus receiving the name *Kocuria rhizophila* and it was the strain that was designated as quality control strain.

Kocuria rhizophila reported in coffee

Kocuria rhizophila have not, for what is known, been found in coffee in earlier studies.

4.2.8. *Micrococcus luteus*

Micrococcus are Gram-positive cocci (Smith *et al.*, 1999) that are 0.5-3.5µm in diameter and are usually arranged in tetrads or irregular clusters, generally they are strict aerobes. They are typically oxidase-positive. They have the ability to aerobically produce acid from glucose glycerol.

Micrococcus luteus can be found in many places as the human skin (Ganz *et al.*, 2003), water, dust and soil. *Micrococcus* are generally thought of to be harmless, but there have been cases of *Micrococcus* infections in people with compromised immune systems. *M. luteus* can be confused with *Staphylococcus aureus* because those two bacteria share similar colony morphology and also similar yellow colony colour, to distinguish the two a bacitracin test can be performed, because *M. luteus* do not grow in the presence of bacitracin.

It is unique because it can absorb UV-radiation (Ganz *et al.*, 2003) and because of this ability cosmetic companies are researching whether or not it can be used as a possible advancement in UV skin protection.

Micrococcus luteus reported in coffee

There have been reports where *Micrococcus luteus* could have been involved (Moreno Cárdenas *et al.*, 2020) in the hydrogen production from coffee mucilage, the mucilage is the layer between the hard, outer skin and the hull of the coffee bean, and they seemed to manage this production through fermentation process from the coffee mucilage. And it also seemed that *M. luteus* was present from the beginning.

4.2.9. *Mycobacterium llatzerense*

M. llatzerense is a Gram-positive bacillus (Gomila *et al.*, 2008), irregular with frequent V forms during cell division. It is aerobic, non-fermentative. The colonies are non-pigmented and growth occur between 22 and 30°C, no growth have been observed at 37°C or at 45°C. It is rod-shaped (Barberán *et al.*, 2017) and non-motile.

There are first and foremost two types of *Mycobacterium* (Delafont *et al.*, 2017) that are highly pathogenic, but those are *M. tuberculosis* and *M. leprae* the other species are called nontuberculous *Mycobacterium*, NTM, and those are ubiquitously found in soil and water. Nowadays it is recognized that several species that are NTM can be human pathogens when transmitted through water and therefore occurrence of *Mycobacterium* in water systems are of particular concern. There have been reports when NTM is associated with Free-Living amoebae, FLA, and *M. llatzerense* is one of the species that are associated with FLA and it has also been reports of *M. llatzerense* in immunocompromised patients and thus raising the possibility that it might be an opportunistic pathogen as well.

Mycobacterium llatzerense reported in coffee

For the bacteria *M. llatzerenze* no reports could be found if it has been found in coffee earlier and therefore this might be the first time it was found in coffee.

4.2.10. *Paenibacillus cookii*

P. cookii got the name from James Cook (Logan *et al.*, 2004a).

Paenibacillus cookii is a Gram-negative (Barberán *et al.*, 2017), rod-shaped bacterium, it is non-motile and the size of the cell is 0.7µm wide and 3.25µm long, it shows halophilic abilities since it can grow in up to 7% NaCl with an optimum of 6% NaCl. It is facultative anaerobe and has the ability to form spores. Even though *P. cookii* is a Gram-negative (Paenibacillus, 2015) bacterium they do have a Gram-positive structure, but in laboratory environment they show to be either Gram-variable or Gram-negative.

The endospores are formed within 2-3 days of incubation in 30°C (Logan *et al.*, 2004a). the spores are ellipsoidal, subterminal located and swells the sporangia slightly. The cells occur singly or in pairs. After 2 days of incubation in 30°C the colonies are 1-4 mm in diameter, convex, yellowish in colour and transparent with opaque centres, some microcolonies are formed and spread across surface of the agar, rotating both clockwise and anticlockwise. Minimum temperature of growth is between 15 and 20°C and maximum temperature for growth at 50°C.

Paenibacillus cookii reported in coffee

There has been evidence for the presence of *Paenibacillus* in coffee leach from coffee machines (Vilanova *et al.*, 2015), but it was not stated which specific *Paenibacillus* that was present in the study made, but it can therefore be stated that this genera have been found in coffee before.

4.2.11. *Paenibacillus yonginensis*

Paenibacillus yonginensis is a Gram-positive (Sukweenadhi *et al.*, 2014), rod-shaped, aerobic bacterium, it has a peritrichous flagella and is thus motile. The vegetative cells are about 0.7-0.9µm in width and 3.4-3.7µm in length. The colonies that are formed after 48h at 30°C are white, round, smooth, raised in middle and are about 2-5mm in diameter. Temperature range of growth is 15-40°C. The type strain was isolated from soil.

P. yonginensis have been inoculated into *Arabidopsis thaliana* as a plant growth-promoting bacteria (Sukweenadhi *et al.*, 2015) and proven to increase tolerance for salt stress, drought stress and heavy metal, aluminium, stress. There is also evidence that the growth parameters as well as the germination ratio was increased with the inoculation of *P. yonginensis*, but the germination did take longer time with inoculated *P. yonginensis*. Plant growth-promoting bacteria forms specific symbiotic relationships with plants, it consists of bacterial strains isolated from diverse environments and that are able to beneficially affect several parameters of plant growth and yield, either directly or indirectly.

Paenibacillus yonginensis reported in coffee

There has been evidence for the presence of *Paenibacillus* in coffee leach from coffee machines (Vilanova *et al.*, 2015), but it was not stated which specific *Paenibacillus* that was present in the study made, but it can therefore be stated that this genera have been found in coffee before. Therefore, the same statement is made for *P. yonginensis* and *P. cookie*, that it cannot be stated if this specific bacterium was present only the genera *Paenibacillus.*

4.2.12. *Staphylococcus epidermidis*

The cells of *Staphylococcus* are characterized as spherical (Zhou, 2015), 0.5-1.5µm in diameter, they lack flagella and are thus non-motile. The cells appears either singly, pairwise, tetrads or in clusters, but they tend to form botryoid clusters and they usually grow in temperatures ranging from 18°C to 40°C. *Staphylococcus epidermidis* is a Gram-positive (Parisi, 1985) and coagulase-negative staphylococcus. *S. epidermidis* is facultative anaerobe (Zhou, 2015) but grows well also under aerobic conditions. The colonies are round, raised, shiny, grey and the edges are complete, they are about 2.5mm in diameter.

When *S. epidermidis* is compared with other bacteria, such as micrococcus, the cell wall is much stronger (Parisi, 1985). It can grow using glucose anaerobically and with oxygen present most strains can produce acid when, for example, exposed to glucose, fructose, maltose and sucrose. It is also the most abundant staphylococcus on human skin.

S. epidermidis typically lives on human skin and mucosa (Presterl *et al.*, 2007); (Zhou, 2015). It is one of the five (Duggirala *et al.*, 2007) most common organisms that cause nosocomial infections because of increased usage of biomaterials in clinical environment. An issue with *S. epidermidis* is if all strains cause disease in an equal way and that is one aspect that need to be further investigated (Mack *et al.*, 2007).

Even though *S. epidermidis* is a colonizer of the skin (Otto, 2009) it has not evolved to cause diseases and it does not produce aggressive virulence determinants and also the role of *S. epidermidis* seems to be balancing the epithelial microflora and serving as reservoir of resistant genes.

S. epidermidis is regarded as an opportunistic pathogen (Otto, 2009) and is the organism that have the most cases of nosocomial diseases. This is likely due to the fact that it is a permanent and ubiquitous colonizer of human skin, but at the same time they rarely develop into life-threatening diseases, the frequency and the extreme difficult of treating leads to a burden for public health system.

It has been proposed that *S. epidermidis* can have probiotic properties (Otto, 2009) by preventing more pathogenic bacteria, such as *Staphylococcus aureus*, to colonize. It is not generally accepted as an enterotoxin producer, but there has been described strain-specific enterotoxin producing *S. epidermidis*.

S. epidermidis has shown resistance towards different kinds of antibiotics (Otto, 2009) for example; methicillin, rifamycin, gentamicin and chloramphenicol, this is most likely due to the fact that resistance against methicillin and other antibiotics is frequent among endemic nosocomial strains, also the ability of some strains to form biofilms decreases the activity of antibiotics significantly. The resistance against antibiotics do reflect the general overuse of different antibiotics.

Staphylococcus epidermidis reported in coffee

In one study the antimicrobial effect of extract from *Coffea arabica* was measured (Runti *et al.*, 2015). The study showed that the extract was effective against *S. epidermidis* and because of the inhibitory effects that coffee extract had on *S. epidermidis* it might be used as a component for hand-washing preparations in healthcare units or more general as a source for antimicrobial agents.

In the case of the study mentioned the extract from *Coffea arabica* had an inhibitory effect on *S. epidermidis* meanwhile in this study one of the identified microorganisms was *S. epidermidis.*

4.2.13. *Staphylococcus hominis*

Staphylococcus hominis have gotten its name from human (Kloos & Schleifer, 1975), the host that it is most commonly found on.
S. hominis is Gram-positive (Kloos & Schleifer, 1975), coagulase-negative cocci, that is non-motile and non-spore forming, the cells are 1.0-1.5 in diameter and they occur mostly in tetrads and occasionally as pairs. The colonies are small, circular, smooth and opaque with an elevated centre those that are opaque do have a yellowish colour. The colonies are 3-4.5mm in diameter. *S. hominis* is somewhat facultative anaerobic but in most cases aerobic. Even though it is aerobic it produces very strong acid rapidly from glucose aerobically. The temperature range of growth is over 15°C, since no growth occur at 15°C, and there is good growth at 45°C.

S. hominis does not, like other coagulase-negative staphylococci, usually cause human diseases (Jiang *et al.*, 2012), but has increasingly been recognized as an opportunistic and nosocomial pathogen that might cause diseases in patients with abnormally weak immune systems.

To be able to distinguish *S. hominis* from other *Staphylococcus* the combination of the colony morphology (Kloos & Schleifer, 1975), pigment pattern and the tetrad formations of the cells is the crucial factors.

Among coagulase-negative staphylococci *S. hominis* is one of the three most frequently found isolates (Mendoza-Olazarán *et al.*, 2013) that have been recovered from blood in immunosuppressed patients and has been associated to be a cause of bacteraemia, septicaemia and endocarditis. Nosocomial infections caused by coagulase-negative staphylococci are associated with the indwelling of medical devices in combination with biofilm-forming potential. *S. hominis* are not typically categorized as a major biofilm producer.

The major pathogenicity factor of most coagulase-negative staphylococci, including *S. hominis*, is the fact that they can form biofilms (Szczuka *et al.*, 2015), but although that most of *S. hominis* carries the *ica* operon that is needed for biofilm production not all strains produces biofilms. Nowadays biofilm-associated infections are of particular concern due to difficulties to eradicate them using standard antibiotics or for them to be cleared by host defensive mechanisms. Regarded the fact that not all *Ica* operon positive strains do produce biofilm this can be explained by the fact that it can be regulated by other genes and also depends on several environmental conditions. The environmental conditions are for example glucose, ethanol, temperature, osmolarity and growth in anaerobic conditions.

4.2.14. *Staphylococcus hominis* subsp. *novobisepticus*

The name novobisepticus derives from (Chaves *et al.*, 2005) the combination of *novobio*, that references the novobicin resistance, and *septicus*, referring to the ability to induce sepsis.

S. hominis subsp. *novobisepticus* colonies are unpigmented (Kloos *et al.*, 1998), greyish-white, opaque, slightly convex and are usually between 5.4-5.8mm in diameter. The optimum temperature for growth is 35°C. It is commonly isolated from human blood cultures, but there have been no reports about isolation from human skin. The type strain of *S. hominis* subsp. *novobisepticus* was isolated from human blood culture. It has spherical cells, that are about 1.2-1.5µm in diameter, and occurs usually in tetrads and sometimes in pairs.

In 1998 the characterization of subspecies *novobisepticus* (Ahmed *et al.*, 2017) was made and thus dividing *S. hominis* into two subspecies; *hominis* and *novobisepticus,* SHN. SHN is reportedly exhibiting multidrug resistance and therefore leaving narrow therapeutic options.

S. hominis subsp. *novobisepticus* are closely related to *S. hominis* (Kloos *et al.*, 1998) strains isolated from human skin, but are significantly divergent. *S. hominis* subsp. *novobisepticus* can be distinguished from *S. hominis* by the combined characteristics of resistance towards novobicin and failure to produce acid aerobically from D-trehalose. It should be mentioned that the bacterium earlier known as *S. hominis,* got the name *S. hominis* subsp. *hominis* when *S. hominis* subsp. *novobisepticus* was identified.

S. hominis subsp. *novobispeticus* is most unusual when it comes to antibiotic susceptibility (Kloos *et al.*, 1998) because it showed to be resistant towards novobicin, penicillin G, oxacillin and streptomycin and at the same time it was either resistant or had intermediate resistance towards methicillin, kanamycin and gentamicin, but all strains was susceptible for vancomycin.

Staphylococcus hominis reported in coffee

What is known for now there have not been any earlier studies where either *S. hominis* nor *S. hominis* subsp. *novobisepticus* have been reported in coffee, naturally or as a contamination bacterium.

4.2.15. *Staphylococcus warneri*

Staphylococcus warneri is a coagulase-negative (Announ *et al.*, 2004) staphylococcus that is a commensally of the skin and represents 1% of the normal staphylococcal population. It is Gram-positive coccus that produces fermentation in presence of sucrose, trehalose, maltose and fructose. It is regarded as an opportunistic pathogen (Władyka & Bonar, 2018).

In food production there is a wide range of staphylococci bacteria present (Władyka & Bonar, 2018) and the diversity of staphylococcal microflora present in fermented meat is often responsible for the uniqueness of the products. *S. warneri* have been found in spontaneously fermented Swiss meat products as the second most abundant staphylococci present during maturation and the final product.

S. warneri has shown to produce molecules with antimicrobial activities (Pauer *et al.*, 2019), those includes the bacteriocin nukacin ISK-1 and warnericin RK. Warnericin RK was the first antibacterial peptide with activity against *Legionella*.

Staphylococcus warneri reported in coffee

There have been one study, that was found, made about the microbial activity when applying the wet method in preparing the coffee beans (Evangelista *et al.*, 2015), this study concluded that *S. warneri* was the most prevalent bacteria in the coffee fruit.

4.2.16. *Bjerkandera adusta*

Bjerkandera adusta is a white-rot fungus (Peralta *et al.*, 2017) that belongs to the family Meruliaceae and is one of six families that are more commonly studied. The white-rot fungi have varying capacities of degrading lignin, cellulose and hemicellulose and thus they are essential part of forest ecosystems. *B. adusta* produces laccases. Laccases are mainly extracellular glycoproteins and attacks the phenolic subunits of lignin. There are three peroxidases involved in the degradation of lignin and those are; Lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP). *B. adusta* is a typical LiP producer but is also a producer of VP. Versatile peroxidase can oxidise both Mn^{2+} and aromatic compounds.

B. adusta has been shown to produce significant amounts of aromatic compounds (de Jong *et al.*, 1994), both in laboratory and natural environment. Veratryl alcohol is the only metabolite (de Jong *et al.*, 1992) that is known to play a role in lignin biodegradation, although many simple aromatic compounds is produced by white-rot fungus. When *Bjerkandera* was grown in synthetic medium with glucose as the only carbon source it produces several aromatic compounds. It produced veratraldehyde and veratryl alcohol, but also anisaldehyde and 3-chloro-anisaldehyde. Veratryl alcohol and veratraldehyde are known compounds produced by white-rot fungi. It was also found that *B. adusta* produces an extracellular aryl alcohol oxidase and this is thought to be an important enzyme in generation of H_2O_2.

Bjerkandera adusta reported in coffee

Studies regarding the presence of *B. adusta* in coffee could not be found at the date when the project was made and thus making this to be, probably, the first time of the identification of *B. adusta* in coffee.

4.2.17. *Neurospora sitophila/Chrysonilia sitophila*

Neurospora sitophila spends most of its lifetime in a vegetative state (Montenegro-Montero *et al.*, 2015), in which it grows as a haploid and multinucleated mycelium. Environmental cues can trigger a developmental program that leads

to the formation of aerial hyphae, followed by segmentation into asexual spores, conidia. Those environmental cues take place in nature every day before dawn as well as under constant conditions, for example under constant darkness or constant temperature.

The genus *Neurospora* contains species with multiple mating systems (Roach *et al.*, 2014), including heterothallism, homothallism and pseudohomothallism, but it is stated that the ancestral state of *Neurospora* is heterothallism and homothallism has evolved independently in sperate lineages. *Neurospora* are able to generate mitotic spores, conidia, that are able to effectively disperse and fill many roles of a sexual spore.

Chrysonilia sitophila is an ascomycete (Ferrer *et al.*, 1992) that belongs to a class of wood-rotting fungi. Some of the potential applications for the lignin peroxidase from *C. sitophila* include biobleaching of wood pulp and degradation of organopollutants. There have been suggestions that usage of these enzymes, especially from basidiomycetes, for biobleaching pulps as an alternative to conventional chlorine-based bleaching.

C. sitophila secretes three extracellular lignin peroxidases (Ferrer *et al.*, 1992) and those have been found to be susceptible to thermal deactivation and the time limit may limit their useful life in enzyme applications.

Neurospora sitophila/ Chrysonilia sitophila reported in coffee

There have been reports about that *N. sitophila* has been identified from coffee ground (Vojtkova *et al.*, 2020) that have been left in coffee dispensing machines. The spores, that are orange coloured, was visible in the coffee ground within a few days. The fungus was found even though the water temperature was up to 85-96°C and therefore it has been noticed as a thermotolerant fungus.

There is one additional case where *N. sitophila* was identified in coffee (Heffler *et al.*, 2009), and in this case the fungus did cause occupational asthma in one patient. The fungus was identified from coffee dispensers. The important information is that if the coffee machines were cleaned daily the fungus did not appear as orange dust on the coffee residuals, but if the machines were not cleaned every day the patient did report respiratory symptoms when cleaning those coffee dispensers.

C. stophila have been reported to cause occupational asthma (Francuz *et al.*, 2010) in a patient that was working as a coffee dispenser operator that mainly was emptying containers of coffee grounds. The patient did show respiratory symptoms when collecting coffee grounds that been stored for longer than 1 week. The coffee was, as with *N. sitophila*, covered in orange powder that was identified as *C. sitophila*. And to prevent the growth of *C. sitophila* the coffee containers should be emptied more often.

5. Discussion

There were microorganisms identified although not every single colony on the agar plates were identified and this could be a source of errors because it is not sure that all microorganisms were the same species and therefore it might have been more microorganisms present in the samples than those that have been identified.

The maximum number of bacteria found was 40 CFU/mL and that was in the sample from cold brewed coffee that was stored at 25°C for 5 days.

The subject that microorganism growth can grow even though the coffee beans have been roasted have not been assessed and this is probably due to the fact that usually the roasting process should kill off the microorganisms' present. Because of this fact the project could not been fully strengthen that the microorganisms found have been present in coffee or not. Some of the identified microorganisms have been found in relation with coffee and those can therefore be provided with support from earlier research that they have been found. There were also species that have not been identified from more than one source in earlier studies, one of them is *Bacillus camelliae* that only have been identified from fermented green tea beforehand. It can also be some explanations to some of the species found that they naturally occur on human skin and because the coffee used had been stored in an open bag for some time beforehand it could have been contaminated if someone grabbed the bag and touched some of the beans used. The bacilli bacteria are usually spore-forming and because of this property they could have survived the roasting procedure and thus they might have been activated when the cold brewed coffee drink was brewed.

Some species are harder to explain why they appeared in the coffee samples and this could either be because that they are present or because of contamination in some stage. The contamination of the cold brewed coffee sample could have happened during the brewing process or during the incubation, because they were stored in a 25°C room at the department. The contamination of the samples from the coffee grinder could have been contaminated during the swabbing, during the transport or when applied to the agar plates.

Some species was also identified from samples that was incubated at 4°C although they should not be able to grow at that temperature, but they could have survived the cooler storing temperature and when the agar plates were incubated at a different temperature that fit their growing range they might have started to grow.

The mould samples were collected from two different agar plates and those two were the only MEAC plates that had growth, what could be seen at least it could have been spores from *N. sitophila* on more plates from the coffee grinder, because this mould seems to be one of the most abundant moulds that can grow in coffee ground.

The presence of bacteria ranged between 2 and 40 CFU/ml and also neither of the more prevalent bacteria, such as *Escherichia coli*, *Staphylococcus aureus* or *Bacillus cereus* was not found and of the identified bacteria seemed to be a human pathogen. The moulds that were identified did not belong to any of the more known human pathogen mould types. *Neurospora sitophila* seemed to be the most prevalent since it has been evidence that it can cause occupational asthma and also that it forms spores when conditions are right and this can be a problem mainly in hospitals and in laboratories. The low presence of bacteria in coffee might be because of antimicrobial activity or that the roasting procedure is effective to kill off most of the bacteria present in unroasted coffee beans.

6. Conclusion

The report did show that it can be growth in coffee beans even though they are roasted and that the hygiene of personnel are of importance since some microorganisms that were identified can be found on human skin. It also shows that cleaning is of importance for example to avoid the formation of *Neurospora sitophila* spores that can cause occupational asthma.

The project showed that the best way of storing cold brewed coffee is at 4°C and in a closed container and for as short period of time as possible, because some growth can still occur. The project did also show that the worst way of storing cold brewed coffee is at 25°C and in open container for longer period of time and this is mainly due to the fact that most microorganisms grow at 25°C and therefore the amount of microorganisms can increase faster than when stored at 4°C.

The project did also show that growth can occur in the coffee grinder and it was mainly *Paenibacillus* that were identified from these samples.

The answers to the research questions at the beginning of this report will therefore be that growth do occur both in a coffee grinder and in cold brewed coffee and most of the growth occur when stored at 25°C in an open container for longer period of time.

The need for more research of this subject is of importance to be able to ensure safe food products and also to gain more knowledge about the roasted coffee beans and that it is possible for microbial growth. This is especially important when it comes to cold brewed coffee since there is no heating steps and that the coffee is stored in a correct way to lower the risk of bacterial contamination. More research generally for roasted, grinded and brewed coffee is needed to gain more knowledge about the microbial growth.

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Appendix 1

Table 9. The type of plate that growth occurred on and on how many of the plates that growth occurred on after incubation time. From those plates colonies was picked and transferred to Nutrient Agar. Samples from coffee grinder was not incubated before they were spread on the agar plates and thus do not have days of incubation before spreading. The samples on MEAC plates was hard to count, because the sample from the cold brewed coffee had grown over the whole plate and looked like one big colony. The sample from the coffee grinder did have a lot of spores and thus it was hard to count how many colonies that was present on the plate. [1]Days of incubation. [2]Inside tube is the inside of the tube where the grinded coffee comes out.

Plate type	Incubation conditions	Amount of plates	Colonies (total count)		
TGEA	Coffee, open, 25°C, 1 day[1]	2 out of 3	15	12	
TGEA	Coffee, closed, 25°C, 1 day[1]	2 out of 3	10	8	
TGEA	Coffee, open, 25°C, 3 days[1]	3 out of 3	24	21	18
TGEA	Coffee, closed, 25°C, 3 days[1]	2 out of 3	17	14	
TGEA	Coffee, open, 4°C, 3 days[1]	3 out of 3	3	2	2
TGEA	Coffee, closed, 4°C, 3 days[1]	3 out of 3	2	1	1
TGEA	Coffee, open, 4°C, 4 days[1]	2 out of 3	8	6	
TGEA	Coffee, closed, 4°C, 4 days[1]	2 out of 3	5	3	
TGEA	Coffee, open, 25°C, 5 days[1]	3 out of 3	40	37	31
TGEA	Coffee, closed, 25°C, 5 days[1]	2 out of 3	28	24	
TGEA	Coffee, open, 4°C, 5 days[1]	2 out of 3	12	9	
TGEA	Coffee, closed, 4°C, 5 days[1]	3 out of 3	8	7	5
TGEA	Coffee grinder, Milling plates	2 out of 3	3	2	
TGEA	Coffee grinder, Inside tube[2]	3 out of 3	2	1	1
TGEA	Coffee grinder, Hand protection	2 out of 3	15	12	
MEAC	Coffee, closed, 25°C, 5 days[1]	1 out of 3	Uncountable, because of growth over the whole plate		
MEAC	Coffee grinder, Milling plates	1 out of 3	Uncountable, a lot of spores		

Appendix 2

Figure 1. Milling plates are circled with red

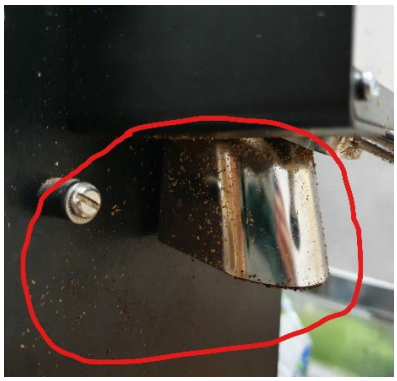

Figure 2. The splash zone is circled with red

Figure 3. The inside of the tube where the coffee come out after grinding

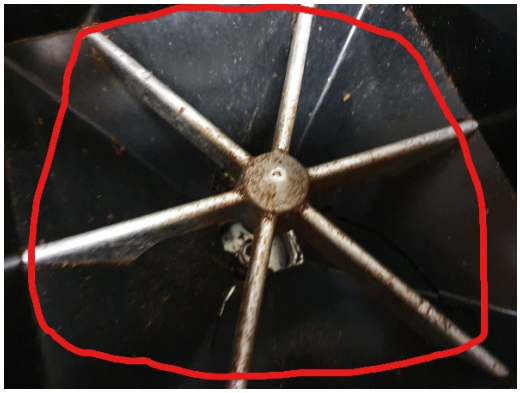

Figure 4. Hand protection is circled with red

Popular scientific summary

Microbial growth in coffee is today an area that almost have been left out from research. In this project the growth of bacteria and moulds in cold brewed coffee beverage, and in the coffee grinders were assessed. The project was therefore about whether or not there can be any microbial growth in cold brewed coffee and in coffee grinder and trying to identify the microorganisms that was found. In this project the first and foremost important question was if there were any microbial growth and therefore the achieving point in this project was to find any microorganism and be able to identify them by using mainly sequencing of their DNA as identification tool and from the sequences the name of the microorganisms could be obtained and further on investigated whether the microorganisms were pathogens for humans or not, in this case most of the microorganisms identified were either spore-forming bacteria, that could have survived the roasting process and been activated when in contact with water, or they were bacteria that exists naturally on human skin and therefore when coffee beans make contact with human skin those bacteria can be transmitted to the coffee beans.

This project is important because it is of importance to know that the coffee that are served is safe to drink and also to gain more knowledge about potential microorganisms that can grow in coffee, since this is not a broad research area from the beginning.

The project was first and foremost directed to companies producing coffee machine and coffee grinder as well to coffee shops that are serving cold brewed coffee beverages and they would care because they have to serve safe products and to know what the best storing conditions are to avoid microbial growth in the products.

The affect that this project will cause is hopefully that coffee shops will think more about the hygiene when preparing coffee and also about the storing conditions of cold brewed coffee. That it can be stored for longer period of time in a closed container of some sort and also if the container is stored in refrigerator. To knowing how beverages and foods should be stored is of importance since this will affect the shelf life of the products. It may also affect the industry that produces for example coffee grinder to see if they can be cleaned in a better way more often than when the milling plates are worn out, and in this way decrease the possibility for microbial growth.

The first applications of this project should be in coffee shops where they serve cold brewed coffee. For example that the coffee will have longer shelf life when stored at 4°C and in a closed container than in 25°C and in an open container, as well as maintain their hygiene so that the coffee beans used will not be contaminated and also the handling of the ground coffee so that if you touch it with your hands the microorganisms that is present on your hands will be transferred to the coffee. Since there is no heating step when making cold brewed coffee those bacteria can survive the brewing process and therefore the hygiene of persons handling the grinded coffee is important.

This report was one of the first to assess microbial growth in cold brewed coffee beverage and therefore more research is needed in this area before any applications may be seen, this is because it is never enough with one study and it therefore need to be assessed more times, with different methods and more precision to obtain result that can resemble what was reached in this project.