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Master Thesis

Evaluation of detection methods for *Alicyclobacillus spp*, aerobic mesophilic microorganisms and yeasts and moulds

by

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Preface

This is the report of my master thesis, my final step at the engineering program in biotechnology at Lund University´s Faculty of Engineering.

As my master´s is in Food Technology and Nutrition, this project involving the fruit juice industry and microbiology was a great opportunity. I have an interest in product safety and quality, which is highly relevant for this project. I have learned a lot about how to plan and execute a project, as well as re-planning. And I learned that nothing ever works out how you want it to. But it will all work out, even during a pandemic.

Best regards, Ella Lagerwall July 10th, 2020

Acknowledgement

I could not have finished this project alone, and have been lucky enough to receive help and support from many people around me.

I would like to thank to thank my examiner, Peter Rådström, for taking the time to read my report and asking me challenging questions during my presentation.

My supervisors, Jenny Schelin and Stina Andren have helped me from start to finish. Plans have changed, and changed again due to certain global events, but with their help I have made it to the end of my time at LTH. Stina and Jenny showed me different points of view, the academic and research from Jenny and the view of a producing company from Stina. Jenny has supported and encouraged me every time I needed a piece of text read through, structure of my work arranged or just a few encouraging words.

My amazing partner, Daniel Espinoza, have supported me with encouragement, proof-reading of the report and a lot of the cooking to allow me to write more. We were able to move in to our new home, at the same time as I were studying for re-exams and started my masters thesis. Alone, this would have taken a lot more of my energy and time.

I also want to thank my wonderful friends, I don´t have the space here for mentioning all of you, but I have some extra special people that really helped during this project. Emma Kihlberg and I have been discussing our challenges with our respective projects and shared our stress. Max Viklund helped me with some translations from german, as well as distracting me at times when I needed to get out of my apartment for some time. Frida Heskebeck, that contributed with her beautiful LaTex template, making this report looking its best.

Thank You!

Abstract

For the fruit juice industry, control of microbial growth is important to keep a high quality and safety of products. Several kinds of microorganisms are important to consider. This project focused on *Alicyclobacillus spp.*, a gram-positive, spore-forming, strictly aerob spoilage bacteria able to grow at low pH of 3-6 and the spores survive pasteurization of 86–96 °C and 2 min. The project also concerned mesophilic aerobic microorganisms and yeasts and moulds.

Several methods for detecting and enumerating these microorganisms were evaluated. Methods from IFU (International Fruit and Vegetable Juice Association), NMKL (Nordic Committee on Food Analysis) and several in-house industry methods were used, as well as Compact Dry plates and the PCR-based Veriflow method. Due to issues with linearity and over growth of agar plates, few comparable results were achieved, and the results were based on other factors: Incubation time, theoretical recovery and simplicity.

For *Alicyclobacillus spp.*, the IFU method and the Veriflow method was deemed the most efficient in terms of time and specificity. For yeast and moulds, the in-house method shows the most promise due to the short incubation time and comparable results to the NMKL-method. For mesophilic aerobic microorganisms the NMKL method and Compact Dry TC was deemed the most efficient.

Sammanfattning

Inom fruktjuiceindustrin är kontroll av mikrobiell tillväxt viktigt för att hålla en god kvalité och säkerhet i produkterna. Flera olika typer av mikroorgnismer är intressanta att ha i åtanke. Detta projekt fokuserade på *Alicyclobacillus sp.*, en grampositiv, sporbildande och strikt aerob bakterie som tillväxer vid låga pH mellan 3-6 och sporerna kan överleva den vanliga pastöriseringen för industrin, 2 minuter och 86-96 °C. Projektet fokuserade också på aeroba mesofila mikroorganismer och jäst och mögel.

Flera olika metodet för att detektera och kvantifiera dessa mikroorganismer var utvärderade. Metoder från IFU (International Fruit and Vegetable Juice Association), NMKL (Nordic Committee on Food Analysis) och fler interna industrimetoder. Dessutom användes Compact Dry plattor och den PCR-baserade metoden Veriflow. Då problem med linjäriteten och och överväxt på agarplattorna uppstod, så gav försöken få jämförbara resultat. Resultaten baserades på andra faktorer: inkubationstid, teoretisk funktion av substraten och hur enkla metoderna var att använda.

För *Alicyclobacillus spp.* var IFU-metoden och Veriflow-metoden de mest effektiva med avseende på tid och specifiktivitet. För jäst och mögel var den mest lovande metoden den interna, då inkubationstiden var kort och substratet jämförbart med NMKL-metoden. För de mesofila aeroba mikroorganismerna var IFU-metoden och Compact Dry TC plattorna de mest effektiva.

Contents

1 Introduction

For the production of foods and beverages, many kinds of microorganisms can create more or less challenging or dangerous issues. Some of them, the food-borne pathogens, can cause illnesses, while a wide array of microorganisms, the spoilage bacteria, do not make us ill but instead negatively impact the quality of the food. The products they grow in develops an undesired taste, smell and consistency. The products are not dangerous to consume, but still cause sever issues with the quality of the products leading to consequences such as food waste, economic losses and the reputation of the company brand put at stake.

Each branch of the food industry is challenged with different microorganisms that affect the safety and quality of the respective products in different ways. For the fruit juice industry, one common genus of spoilage bacteria, *Alicyclobacillus*, can ruin large batches of product if not detected in time. There is a large need in industry to evaluate methods for the for early, rapid, reliable and sensitive detection of this bacterium, as well as collect more knowledge about it regarding for example growth behaviour, spore germination conditions, preferred growth matrices and survival. This will be addressed in this master thesis project. In addition, this project also includes an evaluation off various methods for detecting and quantifying yeasts and moulds and the total count of microorganisms in fruit juice samples.

This report will include background information and theory about the characteristics, environmental conditions and origins of *Alicyclobacillus*. An evaluation of the analytical methods tested for the detection of the bacteria will be included, as well as a detailed suggestion of future work to expand on the subject.

1.1 Aims:

The aims of this project are:

- to study the origins and characteristics of *A. acidoterrestris* in food production and preventative measures to avoid contamination.
- to identify which parameters are necessary for growth of vegetative cells and germination of spores of *A. acidoterrestris* with litterature studies.
- to compare the following analytical detection methods: (IFU (International Fruit and Vegetable Juice Association) standards, NMKL (Nordic Committee on Food Analysis), Veriflow and Industrial in-house methods), for detection and quantification of *Alicyclobacillus spp.* and *A. acidoterrestris* in a systematic way.
- to perform a comparative assessment of in-house, NMKL, Compact Dry and IFU methods for quantifying yeast, moulds and total count. The methods should be as effective as possible regarding to cost, time and specificity.

2 Background

This chapter contains background for the report, including information about spoilage microbes in fruit juices, methods for detection and enumerating microorganisms in foods as well as a introduction to the production of fruit drinks.

Fruit drink production starts with the arrival of either whole fruits, fresh or frozen, juice concentrates or fruit purees at the production facility. The fruit and/or fruit products are stored in tanks, ambient, refrigerated or frozen storage before production begins. The whole fruits are turned into juice or puree, before being mixed with any other ingredients such as preservatives, sugar and flavourings, or just kept like pure juice. The fruit drink is then pasteurized and packaged aseptically in sealed packaging, either hot-filled in glass bottles or in sterile cartons. After the packaging, the beverages are stored either refrigerated or in ambient temperature (The company, 2019).

Potential safety and quality issues can arise in several ways. The largest potential risk is a failure in the pasteurization step. This is a critical step in the production process which ensures product safety and microbial stability. If the product contains spoilage or pathogenic microorganisms that are not reduced to an acceptable level due to pasteurization failure, these can start growing in the packaged product and spoiling it.

2.1 Spoilage microorganisms in fruit juices

Few bacteria can thrive in the low pH environment found in fruits, see table 1, despite fruits having a high water activity. Most spoilage microorganisms needs a water activity (a_w) of at least 0.9 to thrive, which is easily reached in fresh fruits as most fruit have an $a_w > 0.98$ (Michael P Doyle, 1997). Apart from a low pH fruits have an outer peel that protects them from microorganisms. Some plants, and microorganisms, also have antimicrobial agents such as phenolic metabolites

and phytoalexins (Martin R. Adams, 2015a).

The flora of fruits and fruit juices is dominated by yeasts and moulds for this reason as many of these species grow well in low pH in contrast to most bacterial species that prefer a more neutral pH. Examples of strains of yeasts and moulds that can be found on fruits are *Saccharomyces*, *Penicillium* and *Aspergillus*, more examples can be seen in section 2.1.2 below. *Alicyclobacillus* is an exception to the rule, as it is a genus of bacteria that can be found on fruits and in fruit beverages (Martin R. Adams, 2015a).

Table 1 – pH values of some fruits commonly used to produce fruit juice and other fruit beverages (Martin R. Adams, 2015a).

Fruit	pH
Orange	3.3 - 4.3
Apple	2.9 - 3.3
Pear	3.4 - 4.7

Since the most critical step of fruit juice production is the pasteurization, the D-values for microorganisms are of interest. The D-value is defined as the time at a given temperature that will decrease a population by 90 %, or the log-reduction of the population. A pasteurization or similar treatment aims to lower the population by 5-6 log reductions (Martin R. Adams, 2015b). The thermal death of microorganisms can be described by the following equation:

$$\frac{dN}{dt} = -cN$$

Where dN/dt is the death rate and N is the amount of viable cells. c is a constant. The D-value can be calculated from:

$$D = \frac{(t_2 - t_1)}{(logN_1 - logN_2)}$$

Where N_1 and N_2 is the populations at the times t_1 and t_2 (Martin R. Adams,

2015b). Examples of D-values relevant for this report can be seen in table 2 below.

Table 2 – D-values of some microorganisms of high relevance in food production. Both *A. acidoterrestris* and *C. botulinum* are spore-forming bacteria and show a much higher heat-tolerance than yeasts and moulds. The D-value varies some between different fruit juices for *A. acidoterrestris*. The range of D-values for vegetative yeasts and moulds is due to the range of species and their differing tolerances.

Microorganism	Fruit juice	D (mins)	Source
A. acidoterrestris spores	Apple	$D_{85} = 56$	(Silva & Gibbs, 2004)
		$D_{90} = 23$	(Silva & Gibbs, 2004)
		$D_{95} = 2.8$	(Silva & Gibbs, 2004)
	Orange	$D_{80} = 54.3$	(Silva & Gibbs, 2004)
		$D_{90} = 10.3$	(Silva & Gibbs, 2004)
		$D_{95} = 3.59$	(Silva & Gibbs, 2004)
C. botulinum	–	$D_{80} = 0.1 - 3.0$	(Martin R. Adams, 2015b)
Yeasts and moulds	–	$D_{65} = 0.5 - 3.0$	(Martin R. Adams, 2015b)

2.1.1 *Alicyclobacillus spp.*

Discovery: *Alicyclobacillus* (or ACB) is a relatively newly discovered genus of gram-positive bacteria, with new species added to the genus as recently as 2018. The first documented case in the industry is in 1982 in Germany. After a long warm period with temperatures over 26 °C, the bacterium was isolated from apple juice with a pH of 3.15. The juice had been stored after aseptic filling with a pasteurization at 80 °C. The apple juice began to have a undesirable taste and smell after a period in storage. The bacteria was successfully isolated and identified as spore-forming, gram-positive and able to grow at low pH and high temperatures up to 60 °C for vegetative cells. The bacterium was determined to be related to *Bacillus acidocaldarius* (Cerny G, 1984).

Later, it was identified as *Alicyclobacillus acidoterrestris* (figure 1), which causes spoilage in fruit juices. It was classified as a *Bacillus* species until the formation of the new genus *Alicyclobacillus* in 1992, after 16s rRNA sequencing showed that it differed substantially from the *Bacillus* genus. The name originates from the high

occurrence of ω-alicyclic fatty acids in the cell membrane (Wisotzkey, 1992).

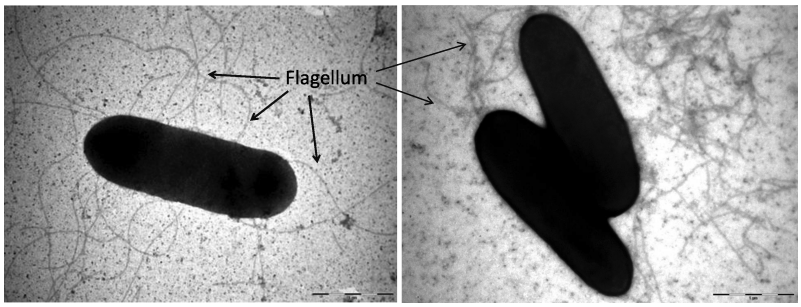

Figure 1 – A transmission electron microscopy photograph of vegetative *A. acidoterrestris* cells, with their flagellum indicated with arrows. The small bars in the bottom right corners indicate 1 µm. Picture reference: (Moshe Shemesh, 2014)

Other species was added to the genus. For example *Alicyclobacillus acidocaldarius* and *Alicyclobucillus cycloheptanicus* which were classified at the same time as the new genus *Alicyclobacillus* with *A. acidoterrestris* (Wisotzkey, 1992). To date 24 species (table 3) have been identified in the genus. Just a few are relevant for spoilage of fruit drinks, namely species isolated from spoiled fruit juices and soil. Most of the spoilage are due to *A. acidoterrestris* but *A. acidiphilus*, *A. herbarius* and *A. hesperidum* has also been found to spoil fruit juices (Clotteau, 2014).

According to a survey, 35 % of spoilage in the fruit juice industry can be attributed to *Acidoterrestris*, which indicates a large issue and potential economic loss for companies (Clotteau, 2014). Most common and closely studied is *A. acidoterrestris*, and therefore this study will focus the most on the specifics for this species (Soisuda Pornpukdeewattana, 2020).

Table 3 – All species in the genus *Alicyclobacillus* to date, including were the species was first isolated from.

Species	First isolated from	Source
A. fastidosus	apple juice	(Goto et al., 2007)
A. acidoterrestris	soil	(Silva & Gibbs, 2004)
A. acidiphilus	acidic beverage	(Matsubara et al., 2002)
A. hesperidum	solfataric soil	(López et al., 2018)

A. sacchari	sugar syrup	(Goto et al., 2007)
A. vulcanalis	hot springs	(Simbahan et al., 2004)
A. sendaiensis	soil	(Tsuruoka et al., 2003)
A. acidocaldarius rittmannii	geothermal soil	(Nicolaus et al., 1998)
A. acidocaldarius acidocaldarius	geyser	(Wisotzkey, 1992)
A. cycloheptanicus	soil	(Wisotzkey, 1992)
A. disulfidooxidans	waste water	(Karavaiko et al., 2005)
A. tolerans	lead-zinc ore	(Karavaiko et al., 2005)
A. pomorum	mixed fruit juice	(Goto et al., 2003)
A. contaminans	orange juice, soil	(Goto et al., 2007)
A. macrosporangiidus	soil	(Goto et al., 2007)
A. herbarius	herbal tea	(Goto et al., 2002)
A. kakegawensis	soil	(Goto et al., 2007)
A. shizuokensis	soil	(Goto et al., 2007)
A. aeris	copper mine water	(Zhang et al., 2015)
A. ferrooxydans	solfataric soil	(Jiang et al., 2008)
A. cellulosilyticus	cedar chips	(Kusube et al., 2014)
A. tengchongensis	hot springs	(Kim et al., 2014)
A. dauci	fruit juice	(Nakano et al., 2015)
A. fodiniaquatilis	copper mine water	(Zhang et al., 2015)
A. montanus	hot springs	(López et al., 2018)

Characteristics: The vegetative *A. acidoterrestris* cells are 2.9–4.3 µm long and 0.6–0.8 µm wide. The spores are 1.5–1.8 µm long and 0.9–1.0 µm wide (Clotteau, 2014). *A. acidoterrestris* is strictly aerobic and grows at a temperature range of 25–60 °C but optimally between 42-53 °C, and at a pH range of 2.2-5.8. Important to note is that *A. acidoterrestris* does not grow below 20 °C, which means that if the product is kept refrigerated, minimal or no growth should occur (Chang, 2004). However, products stored at ambient temperature will be at risk for spoilage. The juices that promote growth of ACB the most are tomato juice and apple juice. Orange, pineapple, grapefruit and pear juices are also susceptible to spoilage (Splltttstoesser, 1994)(Steyn et al., 2011). The growth of vegetative cells is inhibited if the juice has a content of soluble solids of more than 18°Brix, and a pH below 3 inhibits growth severely. Phenolic compounds in for example juice from red grapes could explain a lack of growth in some types of juice. The juices which supported growth of vegetative cells presented with a lower content of soluble solids, mostly sugars. Enough to support growth, but not high enough to inhibit outgrowth of spores. The most important factors for if a juice is susceptible to spoilage by ACB is then a soluble solids content below 18°Brix, a pH between 3-6 and sufficient oxygen (Splltttstoesser, 1994).

The ω-alicyclic fatty acids (figure 2) mentioned above have a crucial role in the stability of the cell membrane of *Alicyclobacillus spp.*. The cell membrane´s fatty acids consists of 15-90 % of ω-alicyclic fatty acids (Hippchen et al., 1981). The fatty acid contains a cyclohexyl-group at the end. This is proposed to lower the fluidity and diffusion of the membranes at high temperatures by being tightly packed and forming a stable layer in the membranes compared to linear fatty acids (Poger, 2015).

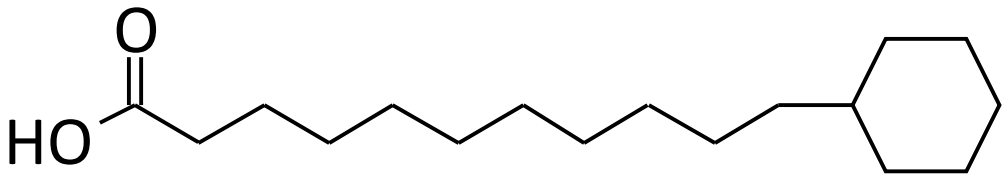

Figure 2 – ω-alicyclic fatty acid, with the cyclohexyl-group on the right end of the carbon chain, and a carboxyl group on the left.

The bacterium originates from the soil, and can contaminate the the surfaces of plant matters, particularly fruit that has not been properly washed. Water contaminated with *A. acidoterrestris* is also a possible route of contamination if used in the production for washing of the fruit or cleaning of equipment (Chang, 2004).

The bacterium is motile with the help of flagella (figure 1) down to about pH 3.6 on certain surfaces, for example on stainless-steel equipment surfaces in production facilities. Some type of liquid, like a juice or water, needs to be present for this to occur. After this, *A. acidoterrestris* starts to form strong biofilms on said surfaces. The bacterium can not become motile again once biofilm has been formed, making the process irreversible. However, spores formed in the biofilm can still be released. Lowering or raising of the pH does not seem to impact the growth rate of *A. acidoterrestris* in any significant way within the optimum range, but mostly affects the motility (Moshe Shemesh, 2014).

No ACB species have been found to have pathogenic properties, after several studies on mice and guinea pigs without any infection showing. However, changes to the product´s taste and smell with ACB growth cause economic losses for producers. All species have in common that they can grow at high temperatures, form spores resistant to common pasteurization temperatures and conditions, and grow well at low pH (Chang, 2004).

Spoilage and guaiacol formation: Spoilage of products by *Alicyclobacillus* species does not produce gas, and is therefore difficult to notice in packaged products. *A. acidoterrestris* produces a few compounds responsible for the changes in taste and smell of spoiled product, mainly guaiacol (see figure 3), which is theorised to be formed from vanillic acid or vanillin with the enzymes vanillin dehydrogenase and/or vanillin oxidase. Some fruits contain vanillic acid and vanillin, which explains the use of this pathway. The exact pathway however seems to be dependent on the strain of *A. acidoterrestris*. Other species of ACB also produce guaiacol, but these pathways are not as closely studied. The sensory threshold for humans to notice guaiacol in a beverage is low, 2 µg/L (Rui Cai, 2015). Guaiacol has a medicinal smell. A few other compounds produced by ACB like 2,6-dibromophenol and 2,6-bichlorophenol add a floral, smokey and sulfuric character to products spoiled by *A. acidoterrestris* (Danyluk et al., 2011).

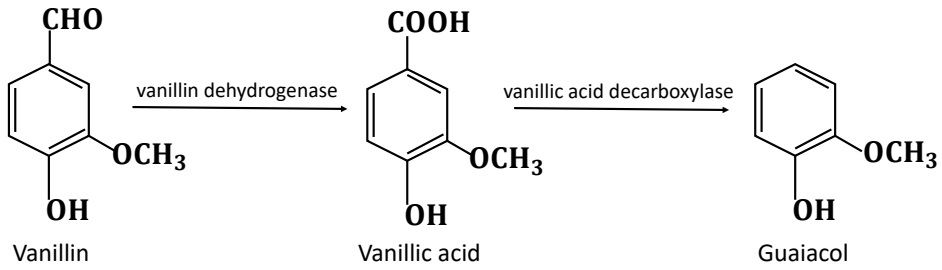

Figure 3 – The pathway of synthesis for guaiacol in *A. acidoterrestris, from vanillin, to vanillic acid and lastly guaiacol.*

Spore formation and control of growth: Spores are dormant, have no metabolism and contain the organism´s DNA inside a hard coating of peptidoglycan. The water content of a spore is very low, which protects it from heat. The production of spores in vegetative cells is triggered by hostile environments for the vegetative cells, such as low amounts of nutrients or sudden temperature changes. When the environment becomes more hospitable, the spore can germinate back to a vegetative cell (Martin R. Adams, 2015b).

The spores are highly tolerant to heat, as seen by table 2. An important factor for this is the mineralization of the spores with cations such as calcium (Ca^{2+}) and manganese (Mn^{2+}). Low pH-values usually demineralize spores, severely lowering their heat-tolerance. In *Alicyclobacillus* species however, the binding of cations is strong, stronger than other spore-forming bacteria like *Bacillus*, allowing the spores to tolerate high temperatures as well as low pH (Chang, 2004).

The industry standard pasteurization of 86–96 °C for approximately 2 min is not enough to inactivate the spores of *A. acidoterrestris*, even if the amount of spores are low. The vegetative cells will however not survive. A raised pasteurization temperature to above 100 °C will inactivate the spores, but this high temperature is not optimal neither for processing conditions nor for sensoric product quality since taste, composition and appearance can be affected by these high temperatures (Danyluk et al., 2011).

The D-value of *A. acidoterrestris* varies with a few factors. The D-values (see table 2) decrease with increasing temperature (85-97 °C) and decreasing amount of soluble solids (5-60°Brix), however changes in pH (2.5-6) do not impact spores (Silva

& Gibbs, 2004).

There are alternative methods of inhibiting growth and preventing spoilage. Before any processing of the fruit, it should be washed thoroughly to avoid contamination of the juice in the first place. Another method is to only keep the juices below 20 °C, or preferably cold-stored, keeping the cold-chain throughout transportation. A promising method is the addition of rosemary extract. The extract is added to apple juice at low concentrations (15-30 µg/mL) at which the addition does not change the taste or smell, but it inhibits the ability for *A. acidoterrestris* spores to grow out as well as the growth of vegetative cells. However, rosemary extract does not deactivate the spores at these low concentrations (Piskernik et al., 2016).

One alternative method that can be used without affecting the quality of the product negatively is ultra-high pressure homogenisation (UHPH), where a temperature of 80 °C and 300 MPa showed up to a 4.8 log-reduction of spores in apple juice. The method of UHPH can also be combined with treatment with short-wave ultraviolet light (UV-C) at doses of 14.3 J/mL. UV-C damages the DNA of microorganisms, making replication of the DNA impossible. With a higher dose of 21.5 J/mL and 60 °C, the method with UV-C can be used very effectively as a single method to inactivate spores (Sauceda-Gálvez et al., 2020).

2.1.2 Yeast and mould

Yeasts and moulds are not as resilient to heat-treatment as *Alicyclobacillus spp.* and other spore-forming bacteria, and the standard pasteurization ised in the juice industry is enough to ensure no growth of either yeasts or moulds (Martin R. Adams, 2015a).

Moulds can grow on a wide array of foods, even foods with a very low a_w like grains and jams, and products with a low pH. Mould can grow on fresh fruits and contaminate food this way, and contamination by air-borne spores in the production facilities are also possible. Moulds are obligate aerobic, and can grow at refrigeration temperatures, but very slowly (Livsmedelsverket, 2017b). Some species of mould commonly found in fruit products are *Aspergillus spp.*, *Penicillium spp.*, *Cladosporium spp.*, *Chaetomium spp.*, *Alternaria spp.* and *Fusarium spp.* (de W Blackburn, 2006a).

Yeasts exist in many places in the environment, such as on the surface of fruits, vegetables and soil. Yeasts can grow at a range of pH 2.5-5. Their need for water is lower than that for moulds, and yeasts can still grow in products with high amounts of sugar and salt, as well as in alcoholic beverages. At pH-values close to neutral, yeasts have difficulties competing with bacteria, but at low pH there are fewer species of bacteria to compete with them (Livsmedelsverket, 2017b). Some species of yeasts commonly found in fruit juices are *Candida spp.*, *Saccharomyces spp.*, *Zygosaccharomyces spp.* and *Picha spp.* (de W Blackburn, 2006b).

2.1.3 Aerobic mesophilic microorganisms

Methods for cultivation of aerobic mesophilic microorganisms are often called the total plate count, and use a general substrate to allow as many types of microorganisms as possible to grow. The incubation temperature of the agar plate is often between 25-30 °C. The results will give an indication of potential spoilage, either from the total microbial load of raw materials or contamination's during production (Livsmedelsverket, 2017a). There are no guidelines or law regulating the amount of CFU/mL (colony forming units) allowed in fruit beverages, instead it is up to each manufacturer to set values. Other foods, however, do have enforced limits. Raw meats, for example, have a limit of $5 \cdot 10^5$ CFU/g (EC, 2005).

A total plate count of aerobic microorganisms will not give any specifics about whether or not the sample contains pathogens, but it is often possible to differentiate moulds, bacteria and yeasts. The aerobic count is an indicator of the microbial status of the raw material, production and environmental condition. A high total plate count does not mean that the product will spoil or is a hazard to consumer health, but it is important to keep the general microbial burden as low as possible to avoid spoilage or growth of pathogens. With continuous testing of products and raw materials, knowledge about the normal level of aerobic mesophilic microorganisms can be established. This will help in discovering potential issues with production or the raw materials (Livsmedelsverket, 2017a).

2.2 Detection and enumeration methods

There are two major different types of methods that can be used for qualification and quantification of microorganisms: The conventional culture based methods, and molecular based methods. This section will explain the differences between, as well as variations and pros and cons of both types. An overview of all methods described can be seen in figure 4. In addition, the methods that are used in this project will be specified.

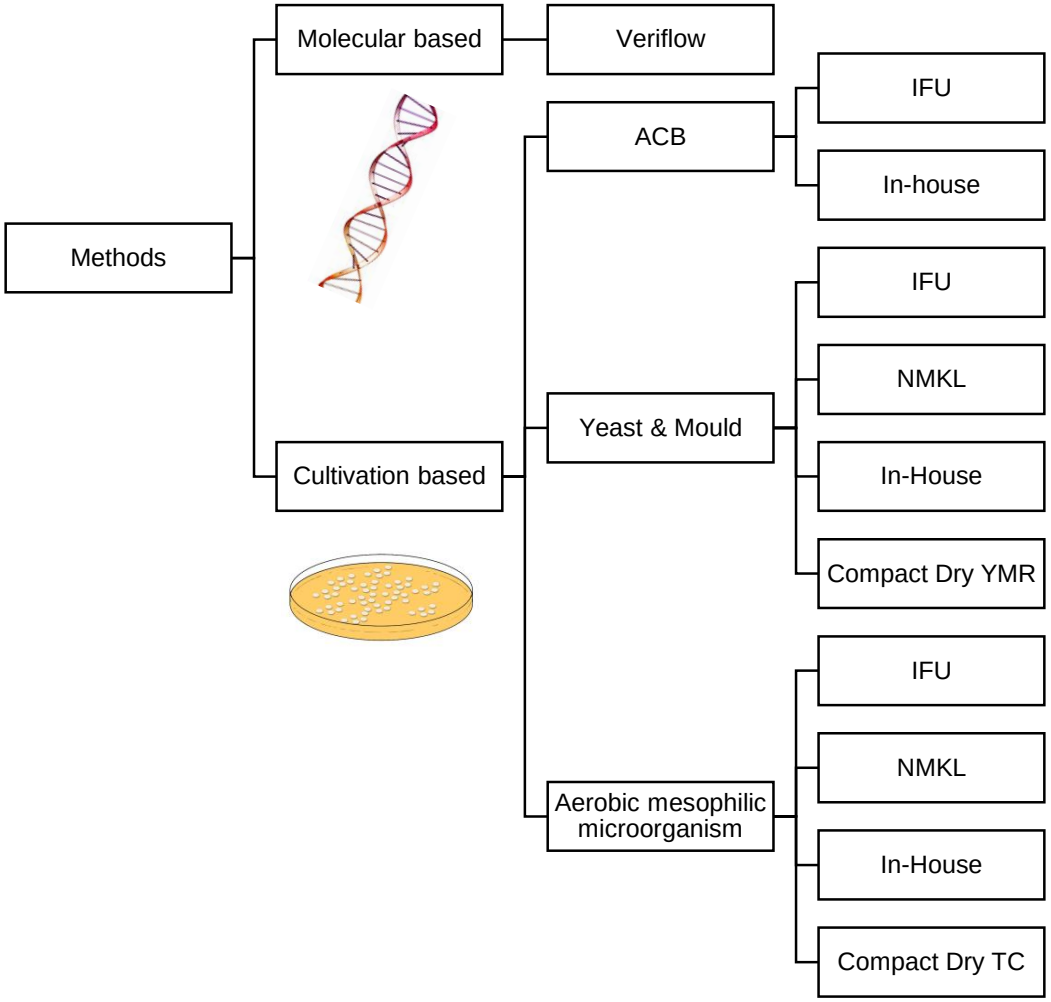

Figure 4 – An overview of all methods used in this project, both molecular based and cultivation based methods, divided by the microorganisms that are targeted.

2.2.1 Cultivation based methods

Substrates: Cultivation based methods often include a substrate with agar in petri dishes, and is very widely used. The methods can be performed with ready-made substrates or the substrates are made in-house. Plates with different forms of substrates can be used, like the plates with freeze dried substrate for the Compact Dry-method described below. In this project several kinds of substrates were used. In the section below all used substrates, their ingredients and other qualities can be seen, and an overview can be seen in table 4 and in figure 5 some agar plates with typical growth for each substrate can be seen. Plate count agar is note included due to the wide array of microorganisms that can grow on this substrate.

Table 4 – List of all types of substrates used, their potential target microorganisms and the methods for which they are used.

Agar	Targeted microorganisms	Used for method
Orange serum agar	aciduric microorganisms	in-house ACB, IFU total count
Compact Dry TC	aerobic microorganisms	total count
Plate Count Agar	aerobic microorganisms	NMKL and in-house total count
BAT	*Alicyclobacillus spp.*	IFU, ACB
OGYE	yeasts and moulds	NMKL, yeasts and moulds
YGC	yeasts and moulds	in-house, yeasts and moulds
Compact Dry YMR	yeasts and moulds	yeasts and moulds

The methods used in this report are based on those used by a few different organizations. The IFU (International Fruit and Vegetable Juice Association) is an organisation that has developed methods for analysing fruit juices and fruit products, striving to create a global standard of methods for the fruit juice industry (IFU, 2020). Two of their methods are used in this project, one for ACB and one for total count of aerobic bacteria. The method for cultivation of yeasts and moulds is mentioned below, but is not used in the project.

The NMKL (Nordic Committee on Food Analysis) works with chemists, microbiologists and statisticians from Finland, Denmark, Sweden, Iceland and Norway. They aim to provide reliable methods to use for microbiological och chemical analyses.

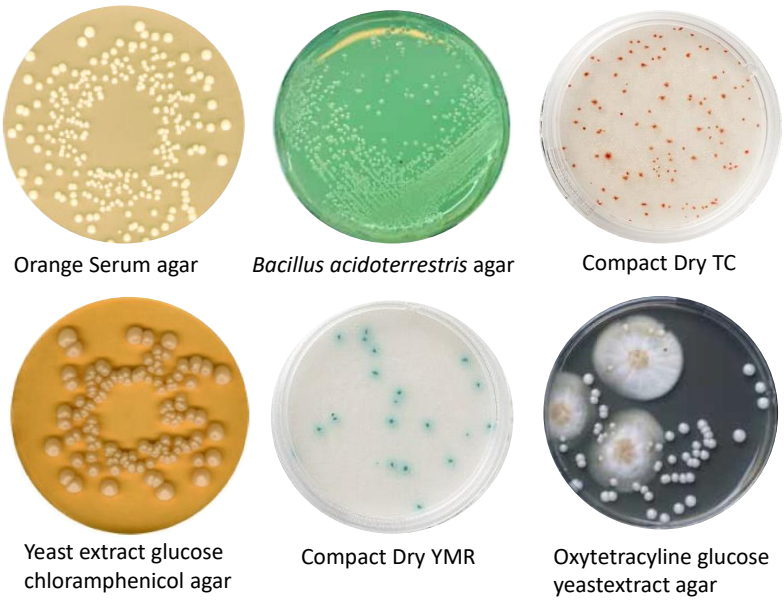

Figure 5 – Examples of plates with typical growth for substrates used in this project: OSA with *Lactobacillus plantarum* (Merck, 2020c), BAT with *A. acidoterrestris* (Merck, 2020a), Compact Dry TC (IWAB, 2020a), YGC with *Saccharomyces cerevisiae* (Merck, 2020e), Compact Dry YMR (IWAB, 2020b) and OGYE with *Candida albicans* and *Aspergillus niger* (VWR, 2020). Note the red and blue color indicators for the Compact Dry plates, indicating colonies of bacteria, yeasts and moulds.

Their methods for cultivation of yeasts and moulds and total count of aerobic bacteria are used in this project (NMKL, 2020).

This project also uses in-house methods from the Company. These are similar to various other methods, for example NMKL.

Cultivation based methods often take 2-7 days before yielding results, which in the food industry is critical time during in which products might need to wait before delivery to costumers. With the right method however, they can be highly accurate, and there are standard methods specifically for the type of matrix of the sample as well as the microorganism targeted. The methods are however often dependent on avoiding contaminants, since a non-selective medium can support growth of microorganisms in the air and surrounding environment of a lab (Martin R. Adams, 2015c).

The type of substrate used for a method is one of the one of the factors that determines which types that can be cultivated on the plate. A substrate can be selective

or general depending on the ingredients. This allows for cultivation of specific types of microorganisms. A common example of how such specificity is achieved is the addition of antibiotics in substrates used for cultivation of yeasts and moulds. The substrate determines the pH and the nutrient profile available for the microorganisms targeted as well. Other important factors are the temperature for incubation of the sample, as well as if the sample is cast inside the substrate (pour plate method) or spread on the surface of the agar plate, allowing different amounts of oxygen to be available for the microorganisms during growth.

A surface spread have more availability to the air than the pour plate method, which can make aerobic bacteria grow easier on the surface of the agar. The effect on the growth seems to vary a lot with the kind of substrate used. A study (Murray et al., 2007) comparing 10 different substrates for growth of *Alicyclobacillus* spores from several species showed that for use of orange serum agar (OSA), the surface plating gave a higher recovery of spores. For *Bacillus acidoterrestris* (BAT) agar however, the plating method did not affect the recovery significantly.

The agar plates can be incubated either right-side up, or bottom up. Bottom up is used for most methods, this stops water to collect on the surface of the agar. For methods expecting growth of moulds, incubating right-side up stops spores from collecting on the lid. These could spread if the plate is opened.

Alicyclobacillus spp. **:** The two cultivation-based methods for for detection of *Alicyclobacillus* in fruit juices for this project can be seen in table 5 below.

Table 5 – Methods used for *Alicyclobacillus spp. during this project, with the incubation time, incubation temperature and substrate listed.*

Method	Incubation time	Incubation temperatures	Substrate
In-house	2+5 days	45 °C	OSA
IFU	5 days	45 °C	BAT

The methods use different agar types, and the in-house method uses heat-treatment and pre-cultivation of the samples to select for spore-forming bacteria, allowing the spores to germinate to vegetative cells in the sample before plating (The Company, 2019). The IFU method also uses heat-treatment to select for spore-forming bac-

teria, but does not pre-incubate the sample. Instead, the sample is plated directly and the spores are allowed to germinate in the substrate on the plate (IFU, 2019).

The agars used for *Alicyclobacillus spp.* are OSA (Orange Serum Agar) and BAT-agar (*Bacillus Acidoterrestris* -agar). Orange Serum Agar, or OSA, contains orange serum, which is made by heating orange juice with a 11°Brix to 93 °C, and filtering it to remove larger particles. The remaining liquid contains fruit sugars, proteins and other compounds from the orange (IFU, 1996). This makes a substrate that creates a similar environment to fruit juices and fruit products, with a nutrients and low pH. Microorganisms such as *Lactobacillus* and *Alicyclobacillus* will grow on the substrate. All ingredients and amounts can be seen in table 6 (Merck, 2020c). Yeast extract contains vitamins, amino acids and carbohydrates, enzymatic digest of casein contains more amino acids and polypeptides. Dextrose is a source of carbon, and the potassium phosphate is a source of phosphates.

Table 6 – List of ingredients for OSA (Merck, 2020c)

Ingredient	Concentration [g/L]
Orange serum	200.0
Yeast Extract	3.0
Enzymatic digest of casein	10.0
Dextrose	4.0
Potassium phosphate	2.5
Agar	17.0

BAT-agar is the type of agar recommended by the IFU for enumeration of *Alicyclobacillus* species. (Murray et al., 2007). An incubation temperature of 45 °C in combination with a low pH of 4 ensures that other microorganisms do not grow. Yeast extract contains vitamins, amino acids and carbohydrates, enzymatic digest of casein contains more amino acids and polypeptides. Glucose is a source of carbon, and the potassiumdihydrogenphosphate is a source of phosphates. The substrate also contains several trace ingredients needed for the growth of *Alicyclobacillus*. A complete list of ingredients can be seen in table 7 (Merck, 2020a).

Table 7 – List of ingredients for BAT agar (Merck, 2020a).

Ingredient	Concentration [g/L]
Yeast Extract	2.00
D (+) glucose	5.00
Calcium chloride	0.25
Magnesium sufate	0.5
Ammonium sulfate	0.2
Potassiumdihydrogenphosphate	3.0
Zinc sulfate	0.00018
Copper sulfate	0.00016
Manganese sulfate	0.00015
Cobalt-chloride	0.00018
Boric acid	0.00010
Sodium molbydate	0.00030
Agar	18.0

Yeasts and moulds: The in-house and NMKL methods for enumeration of yeasts and moulds used in this project uses similar types of media, but with differing antibiotics. Another difference is that the in-house method uses the pour plate technique (The Company, 2019), and the NMKL method uses a surface spread (NMKL, 2015). For this work the IFU-methods for yeasts and moulds were not included or compared with the other methods, due to it being very similar to the NMKL-method, differing only in the incubation time and temperature. An overview of all methods can be seen in table 8.

Table 8 – Methods used for enumeration of yeast and mould in this project.

Method	Incubation time	Incubation temperatures	Substrate
In-house	5 days	25 °C	YGC
NMKL	5-7 days	25 °C	OGYE
IFU	3-5 days	27 °C	OGYE
Compact Dry YMR	3 days	25 °C	-

The Compact Dry YMR is another type of cultivation plate for yeasts and moulds. The plate is smaller than a classical agar plate, and the substrate is dried which enables longer storage. The sample is pipetted directly onto the center of the plate, and allowed to gel with the substrate. If the pH is neutral, or close to neutral, the yeast colonies will appear with a blue colour. The moulds will have a blue background (IWAB, 2020b).

Oxytetracycline-Glucose-Yeast Extract (OGYE) agar (table 9) is a medium used for enumeraion of yeasts and moulds. The medium contains two types of antibiotics (oxytertracycline and gentamicin) to inhibit the growth of bacteria, inhibit the growth of bacteria by inhibiting the synthesis of proteins in bacteria. Yeast extract contains vitamins, amino acids and carbohydrates needed for growth of yeasts and moulds, and glucose provides sugar as a carbon source (VWR, 2020).

Table 9 – List of ingredients for OGYE agar (VWR, 2020)

Ingredient	Concentration [g/L]
Yeast Extract	5.00
D (+) glucose	20.00
Oxytetracycline	0.10
Gentamicin	0.05
Agar	15.0

The YGC (Yeast Extract Glucose Chloramphenicol) agar is used for cultivation of yeasts and moulds. The antibiotic chloramphenicol is used to stop growth of bacteria

by inhibiting the synthesis of proteins. Yeast extract contains vitamins, amino acids and carbohydrates needed for growth of yeasts and moulds, and glucose provides sugar as a carbon source. In table 10 all of the ingredients can be seen (Merck, 2020e).

Table 10 – List of ingredients for YGC agar (VWR, 2020)

Ingredient	Concentration [g/L]
Yeast Extract	5.00
D (+) glucose	20.00
Chloramphenicol	0.10
Agar	14.9

Aerobic mesophilic microorganisms: Four cultivation-based methods were evaluated and compared for the total plate count: 3 methods using substrates with agar, and one using a ready-to-use plate called Compact Dry TC, similar to the Compact Dry YMR described above. For all total count methods, see table 11.

Table 11 – Methods used for total plate count of aerobic mesophilic microorganisms in this project.

Method	Incubation time	Incubation temperatures	Substrate
In-house	5 days	25 °C	PCA
NMKL	3 days	25 °C	PCA
IFU	2-3 days	30 °C	OSA
Compact Dry TC	2 days	30 °C	-

For the In-house method used at the Company, the pour plate method with PCA as cultivation media is performed. As plate count agar (PCA) is used, all bacteria that can grow on a neutral pH in an aerobic environment will grow. For fruit juices this is still mostly yeasts and moulds, and some aciduric bacteria like lactic acid bacteria and *Alicyclobacillus spp.* if the temperature is high enough (The Company, 2019).

Plate Count Agar (PCA), see table 12, is a general (non-selective) cultivation media, designed to allow all aerobic mesophilic microorganisms (Martin R. Adams, 2015c)

that can grow on a neutral pH to do so. It is often used to determine the total plate count of microorganisms, as this can give an indication of the overall quality and microbial content of a sample. The media contains yeast extract which contains amino acids, carbohydrates and vitamins needed for microbial growth. Casein peptone contains proteins and amino acids, glucose is used as a carbon source (Merck, 2020d).

Table 12 – List of ingredients for PCA (Merck, 2020d)

Ingredient	Concentration [g/L]
Casein Peptone	5.0
Yeast Extract	2.5
Glucose	1.0
Agar	18.0

The method proposed by NMKL uses PCA just like the method currently used by the Company, as well as the same pour plate technique. The method suggests a range of temperatures to incubate at, from which 25 °C was chosen. This enables microorganisms that are able to grow close to room temperature to be favoured, as many products are kept at ambient temperatures. A lower temperature requires a longer incubation time, like the proposed 6.5 °C and 10 days. This lower temperature does not favour the expected microorganisms like yeasts and moulds. This could give a misleading result (NMKL, 2013).

The IFU method is designed to favour species of microorganisms that are aciduric and can thrive in the acidic conditions of fruit juices. This includes yeasts, moulds, lactic acid bacteria and other aciduric bacteria like *Alicyclobacillus*. The agar used for this purpose is OSA, see table 6 in the section above (IFU, 1996).

The Compact Dry TC is a cultivation plate for total count of aerobic microorganisms, and works in a similar way to the Compact Dry YMR. If the pH is neutral, or close to neutral, the colonies on the plate will be red (IWAB, 2020a).

2.2.2 Molecular-based methods

Another route for detection and enumeration of microorganisms in food samples is to use molecular based methods. A great advantage over the conventional culture based methods is the expenditure of time, which can be reduced from up to a week for some cultivation based methods down to just a few hours or even shorter for molecular-based methods. A potential disadvantage is that some molecular-based methods do not rely on the viable count of cells, like PCR-based methods, which do not give a good indication of the stage or risk of spoilage. Another disadvantage can be a large initial cost of equipment, like a thermocycler for PCR or equipment for detecting intensity of results. Each run of the equipment can also be more expensive than traditional cultivation-based methods.

One example of a molecular-based method is ATP determination. This method uses the presence of ATP as an indicator, which bound with D-Luciferin produces light which can be detected. All cells contains ATP, and the intensity of the light can be used for determining the amount of cells present. This method is mostly used for monitoring of hygiene status in production facilities. ATP determination can however not differentiate between living or dead cells, due to ATP also being present in dead cells. There are also different types of chemical analyses, in wich volatile metabolites from fungi like *Aspergillus* and *Penicillium* are detected in samples. Most notably, methods based on DNA amplification with PCR of a specific microorganism are becoming more and more available (Martin R. Adams, 2015d). For this project the Veriflow-system, detailed below, was evaluated and compared to the cultivation-based methods.

Veriflow: Veriflow (produced by Invisible Sentinel) is a PCR-based method for identifying *Alicyclobacillus* species in food samples. Similar methods exists for other microorganisms, like *Salmonella*. The method takes in total 3 hours to complete, and has a sensitivity of detection of 10 cells/mL. The PCR-amplification is done with a primer with a tag. The primer is designed for targeting the DNA of *Alicyclobacillus*. After the thermocycling the sample is added to the test window of a cassette used for the test results (see figure 6 for a picture of the cassettes). Here the DNA fragments with the tag are connected to a protein-bound gold particle, which in turn shows the positive result (Invisible Sentinel, 2020a).

Figure 6 – Picture of a cassette from the Veriflow ACB test kit, showing the test window in pink, and the black button slid down to reveal the result (Invisible Sentinel, 2020a).

More DNA fragments give a more intense color, which allows the method to be used as a semi-quantitative method, see figure 7. A more intense test-line indicates a higher concentration of cells in the sample.

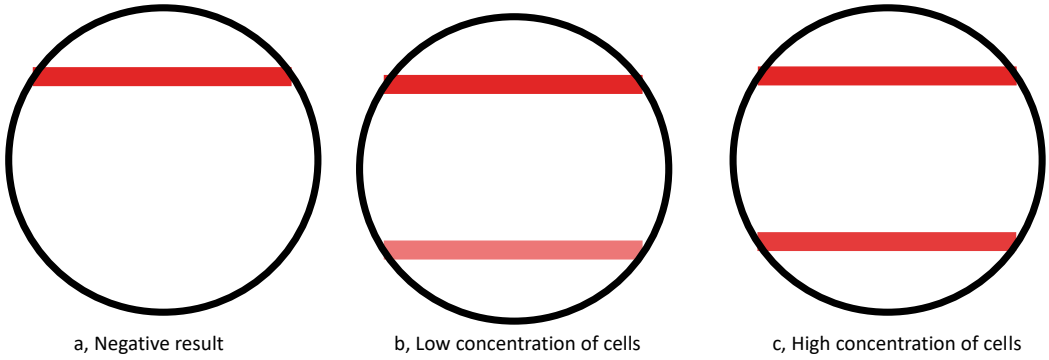

Figure 7 – Schematic of the test windows of the cassettes, showing a, negative result, b, low concentration of cells and c, high concentration of cells result.

A specific reader for the results can be added, making it a more precise quantitative method than just using visual determination. An important note is that the method detects even dead cells, not only viable ones. This might give a misleading result, and if needed the Veriflow should be followed up with a cultivation based method to quantify viable cells (Invisible Sentinel, 2020a).

3 Materials and Methods

3.1 Materials

The microorganisms to be used in the experiments in this project were stored and kept on agar plates in a fridge in the microbiology lab of the company. These species had been isolated and kept from previous in-house tests in the company, and the species of microorganisms was not determined. The juice products, used as matrices in this project, were inoculated with one colony from these plates. In table 13 all types of substrates are listed, together with the usage and manufacturer.

Table 13 – List of substrates used for the project, their usage and the manufacturers.

Substrate	Usage	Manufacturer
Orange serum agar (OSA)	aciduric microorganisms	VWR chemicals
Compact Dry TC	aerobic microorganisms	IWAB
Plate Count Agar (PCA)	enumeration of aerobic microorganisms	VWR chemicals
Bacillus acidoterrestris -agar (BAT)	enumeration of ACB	Merck
OGYE	enumeration of yeasts and moulds	VWR chemicals
YGC	enumeration of yeasts and moulds	VWR chemicals

Compact Dry YMR	enumeration of yeasts and moulds	IWAB

For this project, several fruit juices and fruit drinks were used as matrices. All products, which method they are used for and the main ingredients are listed in table 14. The products were provided by the Company, all were packaged and pasteurized product except for the raw-material apple concentrate.

Table 14 – List of all products used as matrices for this project, the methods in which they are used, as well as their ingredients and storage.

Product	Methods	Ingredients	Storage
Smoothie	ACB-methods	Pineapple 54 % Banana 32 % Coconut milk 9 % Apple Lemon juice	Refrigerated
Ready-to-drink apple drink	Veriflow	Apple juice 20 % Sugar 6.5 % Citric acid Ascorbic acid Natural flavours	Ambient
Raw material apple juice concentrate	Veriflow	Apple juice	Frozen
Ready-to-drink pear drink	Yeast and moulds,	Pear juice 20 %	Ambient

	mesophilic microorganisms	Sugar 6.5 % Citric acid Ascorbic acid Natural flavours	
Pear drink concentrate	Yeast and moulds, mesophilic microorganisms	Pear juice Sugar 27.5 % Citric acid Ascorbic acid Natural flavours	Ambient

A list of the other materials used in this project, the manufacturers and specifications can be seen in table 15 below. In the method description, the products will be mentioned with the product name and manufacturer.

Table 15 – List of all products used as matrices for this project, the methods in which they are used, as well as their ingredients.

Product name	Manufacturer/Source	Specifications
NF 400 Medium Capacity Centrifuge	Nuve (Nuve, 2020)	Maximun RCF: 2.819xg Power supply: 450 W
Water bath, TE-10A Tempette	TECHNE OneMed (TECHNE, 2020)	Capacity: 8 L Stability: ±0.01 °C
Sample beakers	TH Geyer GmbH & co (TH Geyer, 2020)	Volume: 200 mL Max. 100 °C Red screw cap

Centrifuge tube PP with screw cap PE	Nerbe plus (Nerbe plus, 2020)	Volume: 50 mL Green screw cap
Veriflow Thermocycler	Invisible Sentinel (Invisible Sentinel, 2020a)	Capacity: 96 samples DNA-amplification programs
Veriflow ACB test kit	Invisible sentinel (Invisible Sentinel, 2020b)	Contains: ACB PCR Tube ACB buffer Buffer B ACB Assay Cassette 1.5 mL Sample tube

3.2 Experimental design

For each type of microorganism described above, several methods were tested. The methods used for ACB were the IFU-method for ACB, an In-house method for ACB from the Company as well as the Veriflow method from Invisible Sentinel. For yeasts and moulds the In-house method, NMKL-method and Compact Dry YMR plates from IWAB was used. For aerobic mesophilic microorganisms the In-house method, NMKL-method, IFU-method and Compact Dry TC plates was used. An inoculation of juice products were used as samples, and the same samples was used for all methods for each type of microorganism (aerobic mesophilic organisms, ACB and yeasts and moulds) to enable comparison between the methods. The workflow used can be seen in figure 8. An initial sample preparations, was followed by plating and incubation for the cultivation based methods, and preparation of PCR-samples and heat-treatments for the molecular-based method Veriflow.

After the incubation time for the agar plates, the colonies were counted on the plates with 30-300 CFU. For the dilutions that reached this range of colonies, a mean value

of the triplicates were made and the CFU/mL was calculated, see equation below.

$$Mean = \frac{(Plate_1 + Plate_2 + Plate_3)}{3}$$

If no correlation between the dilutions could be seen, the CFU/mL was not calculated. If several dilutions contained between 30-300 CFU, a mean-value between the CFU/mL was calculated.

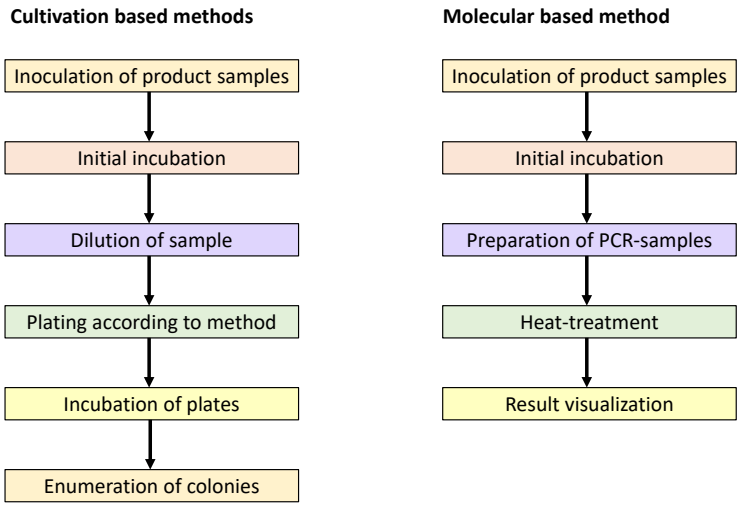

Figure 8 – The workflow used for the project, from the inoculation of juice product samples to the finished results for both the cultivation based methods and the molecular based methods.

3.3 Sample preparations

Inoculation of product: For all methods to be tested for the enumeration of *Alicyclobacillus spp.*, yeasts and moulds and aerobic mesophilic microorganisms, the same preparation procedure was performed for the samples used for evaluating the different methods. Details on which products that were used for the different methods can be seen below. 200 mL of the products were transferred to sterile plastic beakers (Sample Beakers, TH. Geyer GmbH & co).

The fruit juice products used as matrices for this project were inoculated, using a sterile loop, with one colony from one colony from the petri dishes with microorgan-

isms provided by the Company, and then mixed by shaking of the plastic beakers. The samples were incubated at 25 °C for 3 days. The samples were then diluted as described below.

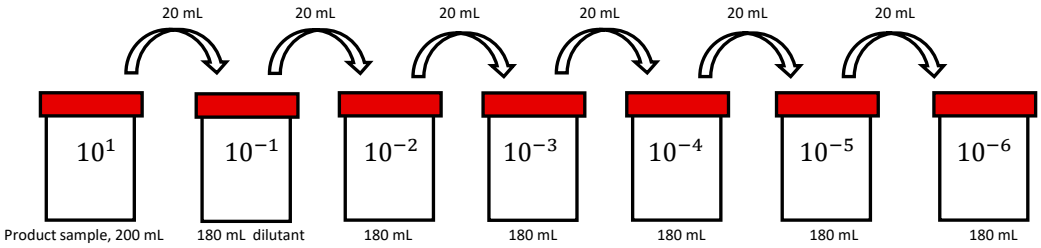

Figure 9 – Schematic over the dilution of samples, where 20 mL of the initial sample is diluted with 180 mL of 0.9 % NaCl-solution, creating the first dilutant of 10^{-1} or 1/10. This is repeated until a desired range of dilutions are received, in this case 10^{-1} to 10^{-6}.

All dilutions were made in the same type of sterile beaker as the inoculation, with sterile 0.9 % NaCl solution. Then 180 mL (9/10 of the total volume) of the NaCl-solution (Sigma/Merck??) was poured into beakers (Sample Beakers, TH. Geyer GmbH & co). Twenty mL (1/10 of the total volume) of inoculated product were added to the first beaker, thoroughly mixed by shaking. This becomes the first dilution of 1/10 of the original sample, or 10^{-1}. Then 20 mL from 10^{-1} were transferred to the next beaker and the procedure is repeated until a desired range of dilutions from 10^{-1} to 10^{-6} is achieved. See figure 9 for a schematic overview of the procedure. Then the dilutions were plated on selective and general substrates according to what organism the inoculation was made with and the different methods. See figure 10 for a figure over the whole sample preparation.

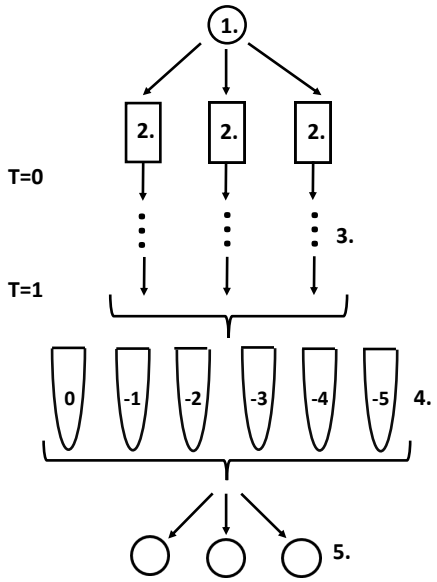

Figure 10 – Overview over the sample preparation. Inoculation from agar-plate (1) into jars prepared with 200 ml fruit juice (2). The samples are allowed to incubate (3) at 25 °C, then the samples are diluted (4) before plating triplicates from each dilution and juice sample (5) according to the respective methods.

3.4 Method descriptions and enumeration methods

3.4.1 *Alicyclobacillus spp.*

For the enumeration and cultivation of *Alicyclobacillus spp.* three methods were used. First, the in-house cultivation-based method used by the company, then the IFU method using BAT agar, and lastly the molecular-based Veriflow method also used by the company.

Two sets of experiments were made for ACB. The first set were made with the smoothie, apple drink and apple juice concentrate. Since issues with the results arose, a second set were made. The second set were made with only the cultivation based methods and a new smoothie sample.

In-house method: The following sample dilutions were used: 10^{-2}, 10^{-4} and 10^{-6}, as the samples were placed in a plastic beaker (Sample Beakers, TH. Geyer GmbH & co) in a water bath (Water bath, TECHNE OneMed) at 82 °C and was held at the temperature for 15 minutes to select for spores. The sample was then pre-incubated in the same beaker at 45 °C for 2 days. OSA agar was melted and poured into 9 cm

petri dishes and allowed to solidify. One mL of the samples were then spread on the surface of the agar, for 3 plates with the 10^{-4} dilution, 0.1 mL were used instead. Triplicates of each dilution were plated. The dishes are put in plastic bags and were incubated right-side up at 45 °C for 5 days. The colonies were counted and noted (The Company, 2019).

IFU method: The following sample dilutions were used: 10^{-2}, 10^{-4} and 10^{-6}, as the samples were placed in the plastic beakers (Sample Beakers, TH. Geyer GmbH & co) in a water bath (Water bath, TECHNE OneMed) at 80 °C and was held at the temperature for 15 minutes to select for spores. The sample was allowed to cool to 20 °C, but was not allowed to sit for more than 45 min before plating to avoid germination of spores. BAT agar was melted and cooled to 45 °C. 2 mL of the samples were added to a petri dish, then 15-20 mL of BAT was added to the petri dish and mixed in a figure 8. Triplicates of each dilution were plated. The agar was allowed to solidify completely, then the petri dishes were put in plastic bags and were incubated bottom up at 45 °C for 5 days. The colonies were counted and noted (IFU, 2019).

Veriflow method: Analysis of ACB using Veriflow was performed according to the instructions from the manufacturer, Invisible Sentinel. Here follows a summary. The following sample dilutions were used: 10^{-2}, 10^{-4} and 10^{-6}. Twenty mL sterile, room-temperature distilled water was pipetted into a 50 mL test-tube (Centrifuge tube, Nerbe plus). Five mL of the sample was added to the tube, the tube was mixed with a vortex. The tube was centrifuged (NF 400 Medium, Nuvefor) for 10 min at 2.819xg. The supernatant was poured out carefully as to not disturb the pellet. One mL of the ACB-buffer provided with the test-kit was added to the tube. The pellet was dissolved in the ACB-buffer with the vortex. Then 50 µL of the sample was transferred to a "sample tube" provided by the Veriflow kit. The tube was mixed with a vortex. Five µL is transferred from the sample tube to the PCR-tube containing the primers targeting ACB species DNA sequences. The PCR-tube was put in the Thermocycler (Veriflow Thermocycler, Invisible Sentinel) and the program "VFLOWACB" was run. After the program was finished, 5 drops of Buffer B (Provided by the Veriflow kit) was added directly to the PCR-tube, and the contents were carefully mixed by knocking the tube against the table. After this, 200 µL from the PCR-tube was transferred to the test cassette window, the

cassette was allowed to rest for 2 min ± 15 s. Four drops of Buffer B was added to the test window, and allowed to rest for 1 min ± 15 s. The black button on the cassette was slid down and the results noted (Invisible Sentinel, 2020a).

3.4.2 Yeasts and moulds

For these methods, the pear drink and pear drink concentrate were used.

In-House method: The following sample dilutions were used: 10^{-1} to 10^{-4} and 10^{-6}. YCG agar was melted and allowed to cool to 45 °C. One mL of the samples were added to a petri dish, then 15-20 mL of YCG was added to the petri dish and mixed in a figure 8. Triplicates of each dilution were plated. The agar was allowed to solidify completely, then the petri dishes were put in plastic bags and were incubated right-side up up at 25 °C for 5 days. The colonies were counted and noted (The Company, 2019).

NMKL method: The following sample dilutions were used: 10^{-1} to 10^{-4} and 10^{-6}. OGYE agar was melted and poured into 9 cm petri dishes and allowed to solidify. Then 0.1 mL of the samples were then spread on the surface of the agar. Triplicates of each dilution were plated. The petri dishes were put in plastic bags and were incubated right-side up at 25 °C for 5-7 days. The colonies were counted and noted. If no growth was noted after 5 days, the samples were allowed 2 more days of incubation (NMKL, 2015).

Compact Dry YMR: The following sample dilutions were used: 10^{-2}, 10^{-4} and 10^{-6}. The lids of the Compact Dry YMR plate was lifted and 1 mL of the samples were added to the center of the plate and allowed to sit for 5 minutes to ensure absorption of the sample. Triplicates of each dilution were made. The plates were put in plastic bags and were right-side up at 25 °C for 72 hours. The colonies were counted and noted (IWAB, 2020b).

3.4.3 Aerobic mesophilic microorganisms

For these methods, the pear drink and pear drink concentrate were used.

In-House method: The following sample dilutions were used: 10^{-1} to 10^{-4} and

10^{-6}. PCA was melted and allowed to cool to 45 °C. One mL of the samples were added to a petri dish, then 15-20 mL of PCA was added to the petri dish and mixed in a figure 8. Triplicates of each dilution were plated. The agar was allowed to solidify completely, then the petri dishes were put in plastic bags and were incubated bottom up at 25 °C for 5 days. The colonies were counted and noted (The Company, 2019).

IFU method: The following sample dilutions were used: 10^{-1} to 10^{-4} and 10^{-6}. OSA was melted and allowed to cool to 45 °C. One mL of the samples were added to a petri dish, then 15-20 mL of OSA was added to the petri dish and mixed in a figure 8. Triplicates of each dilution were plated. The agar was allowed to solidify completely, then the petri dishes were put in plastic bags and were incubated bottom up at 30 °C for 2-3 days. The colonies were counted and noted (IFU, 1996).

NMKL method: The following sample dilutions were used: 10^{-1} to 10^{-4} and 10^{-6}. PCA was melted and allowed to cool to 45 °C. One mL of the samples were added to a petri dish, then 15-20 mL of PCA was added to the petri dish and mixed in a figure 8. Triplicates of each dilution were plated. The agar was allowed to solidify completely, then the petri dishes were put in plastic bags and were incubated bottom up at 25 °C for 3 days. The colonies were counted and noted (NMKL, 2013).

Compact Dry TC: The following sample dilutions were used: 10^{-2}, 10^{-4} and 10^{-6}. The lids of the Compact Dry TC plate was lifted and 1 mL of the samples were added to the center of the plate and allowed to sit for 5 minutes to ensure absorption of the sample. Triplicates of each dilution were made. The plates were put in plastic bags and were bottom up at 30 °C for 48 hours. The colonies were counted and noted (IWAB, 2020a).

4 Results

The product sample used for all methods can be seen in table 16. For the cultivation based methods, the plates with 30-300 CFU was counted on the series of plates with correlations between the dilutions. After this a mean value of the CFU for each dilution and the CFU/mL was calculated. If several dilutions contained between 30-300 CFU, a mean-value between the CFU/mL was calculated.

Table 16 – List of all fruit juices used as matrices for this project, and the methods they were used for.

Method	Products used
ACB-methods	Smoothie
	Ready-to-drink apple drink
	Raw material apple concentrate
Yeast and moulds	Pear drink concentrate
	Ready-to-drink pear drink
Aerobic mesophilic	Pear drink concentrate
microorganisms	Ready-to-drink pear drink

4.1 *Alicyclobacillus spp.*

For the first set of experiments for the ACB-methods, all juices listed above were used as samples. For the second, only the smoothie was used for the cultivation based methods due to material constraints.

IFU: The IFU method with BAT-agar did not show any growth for the first set of plates made. In figure 11, the results from the second set of plates can be seen.

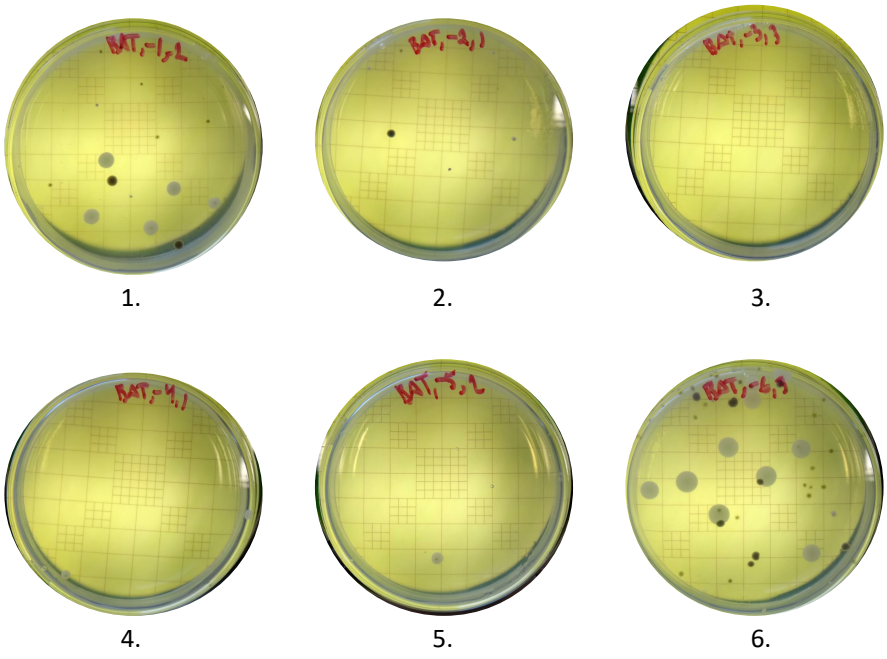

Figure 11 – The figure above shows a collection of plates from the IFU method with BAT agar. One plate out of three for each dilution 10^{-1} to 10^{-6} were chosen and numbered 1-6 in order. On plates 3 and 4 no microbial growth was detected. The other plates shows varying degrees of growth with small, green, fluffy colonies and white, creamy colonies. The white colonies looks like the expected growth of *Alicyclobacillus spp.*.

There was no correlation in the amount of CFU/ml between dilutions as normally is expected. The plates with the dilutions 10^{-1} and 10^{-2} indicates some correlation, but the plates with 10^{-3} and 10^{-4} did not show any growth at all. On the plates with 10^{-5} and 10^{-6}, the amount of colonies were comparable to the first two dilutions. The colonies were both the white, creamy colonies expected by *Alicyclobacillus spp.*, but also green, sometimes fluffy colonies.

In-House: The same juice samples were used for both the IFU method and the In-house method. The plates made with this method, see figure 12, presented with a fluid layer on top of the surface of the agar, making the counting of colonies difficult. This is a known issue with this method, resulting in making the plates impossible to incubate bottom-up. No colonies were present on the plates with 10^{-1} and 10^{-2} dilutions. The rest of the plates showed a too high amount of colonies (above 300) or were impossible to count due to the fluid layer.

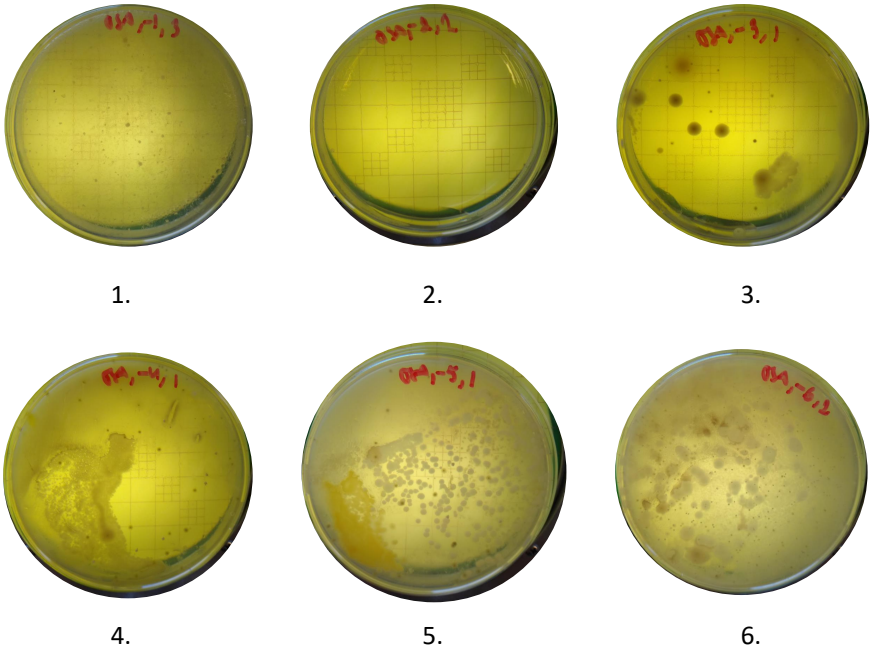

Figure 12 – The figure above shows one plate out of three from the In-house method with OSA agar, with each dilutions 10^{-1} to 10^{-6} numbered 1-6 in order. The first two plates did not show any colonies, but the rest had varying degrees of overgrowth.

Plating with OSA: With the same juice samples as the in-house and IFU method, some tests were made with differing methods for plating on the OSA agar. The recommended 1 mL surface spread were compared to 1 mL sample with the pour plate method, as well as a surface spread with 0.1 mL. On all the plates with 1 mL surface spread, the sample did not absorb to the surface of the agar, making incubating them turned up-side-down impossible without sample dripping down on the lid. With 0.1 mL this did not occur, however it should be noted that this results in an extra dilution of the sample on the plate, compared to 1 mL surface spread. In figure 13, the 0.1 mL sample is from the 10^{-3}-dilution, and the 10^{-4}-dilution for the 1 mL.

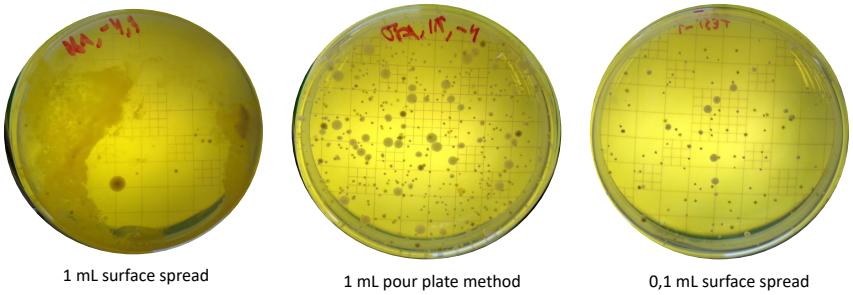

Figure 13 – The petridishes above shows variation of the in-house cultivtionmethod for ACB. In the first petri dish, 1 mL the 10^{-4} dilution of inoculated smoothie sample were spread on top of the agar according to instructions. In the second, 1 mL of sample were added, and agar poured on top with the pour plate method. In the third, 0.1 mL of the sample were spread on top of the agar.

Veriflow: For all Veriflow tests with ACB-inoculated juices the samples were undiluted. The cassettes can be seen in 14 below. The apple concentrate, cassette 2, shows the strongest positive result. The third cassette with apple beverage, showed the weakest positive. The first, with the smoothie sample, showed a bit weaker positive than the second.

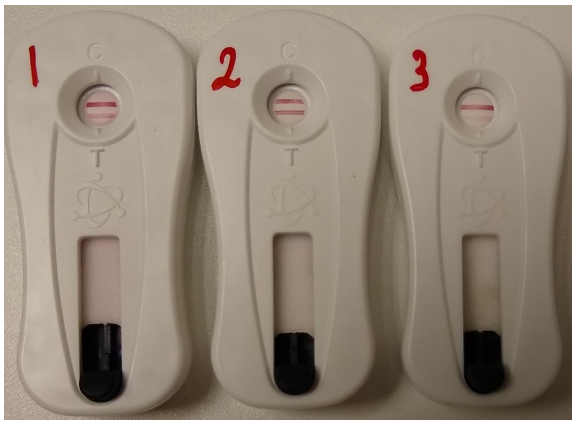

Figure 14 – The results from the Veriflow method. The cassettes are marked with number, were 1 indicates the smoothie sample, 2 apple concentrate and 3 apple beverage. The second cassette show the strongest positive result, then the first, then the third with a weak positive.

4.2 Yeast and moulds

For the methods used for enumerating yeast and moulds, the juice samples used were pear drink concentrate and pear drink. Three cultivation based methods were used: The in-house method, NMKL method and Compact Dry YMR. Some of the plates from this section of the project can be seen in figure 15. The plates with the pear drink concentrate did not show any growth of moulds, which was present on the plates with pear drink. Interesting notes on the plates are that the color indicator did not work well on the Compact Dry YMR, no blue colonies was present. The 10^{-1} dilution showed a pale blue color over the surface of the plate, unfortunately not visible in the photograph.

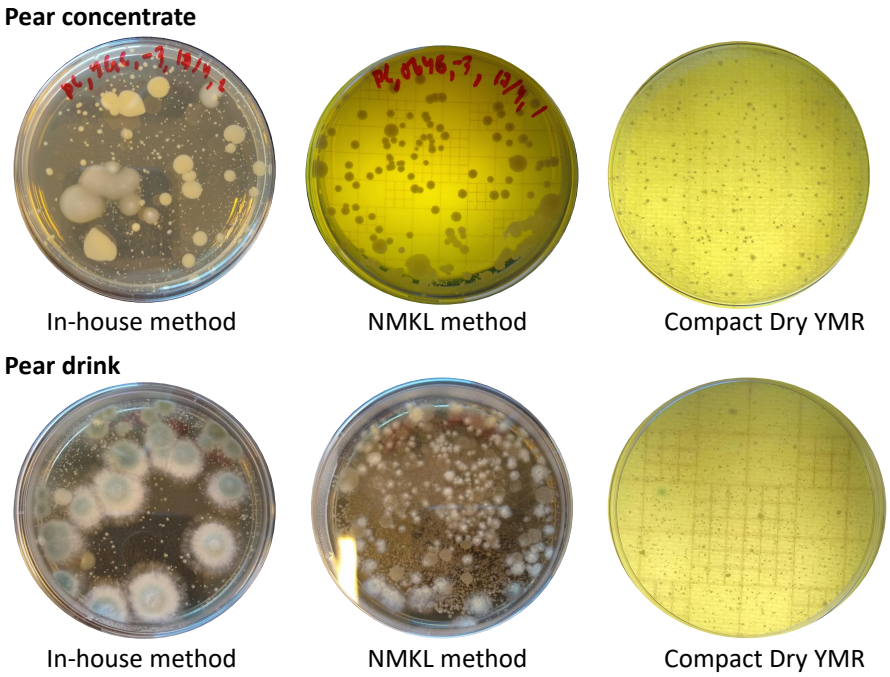

Figure 15 – In the picture above some of the petri dishes from the methods for yeasts and moulds (In-house method, NMKL method and Compact Dry YMR) are shown. The upper three from the samples with pear concentrates, and the lower three from the the pear drink. Generally the differences are the presence of mould for the pear drink, which were not noticed in the pear concentrates.

In table 17 the results for the plates made with the pear concentrate can be seen, as mentioned above these plates did not show any growth of mould. All methods show similar amounts of growth, with the in-house method having a slightly

higher amount. The NMKL method and Compact Dry YMR both had around 10^5 CFU/mL.

Table 17 – The mean-value of the CFU/mL for the yeast and mould method with the pear concentrate samples. The (–) indicates no growth.

Method	Yeast [CFU/mL]	Mould [CFU/mL]
In-house method	$1.5 \cdot 10^6$	–
NMKL method	$1.4 \cdot 10^5$	–
Compact Dry YMR	$3.5 \cdot 10^5$	–

In table 18 the results for the plates with the pear drink can be seen. All plates had both yeast and mould growth. The in-house method and Compact Dry YMR gave very similar results for the yeast count. However, the mould count differed a bit for these. The NMKL method had over 300 CFU of yeasts on all plates, but the count of moulds were similar to both the in-house method and Compact Dry YMR.

Table 18 – The mean-value of the CFU/mL for the yeast and mould method with the pear drink samples. TNTC=Too numerous to count.

Method	Yeast [CFU/mL]	Mould [CFU/mL]
In-house method	$2.6 \cdot 10^6$	$4.7 \cdot 10^4$
NMKL method	TNTC	$3.0 \cdot 10^3$
Compact Dry YMR	$2.3 \cdot 10^6$	$3.0 \cdot 10^2$

4.3 Aerobic mesophilic microorganisms

For the enumeration methods for aerobic mesophilic organisms, the juice samples used were pear drink concentrate and pear drink. Three cultivation based methods were used: The in-house method, IFU method, NMKL method and Compact Dry TC. In figure 16 a selection of the agar plates from the methods of aerobic mesophilic microorganisms can be seen. Both juice samples showed growth of both moulds and other colonies. Mostly white, but as can be seen for the in-house method and pear concentrate, yellow and creme colored colonies were present on the plates as well. The color indicator did not work for the Compact Dry TC. No red colonies except for one colony on the 10^{-6} dilution for the pear drink, which showed a single, red colony.

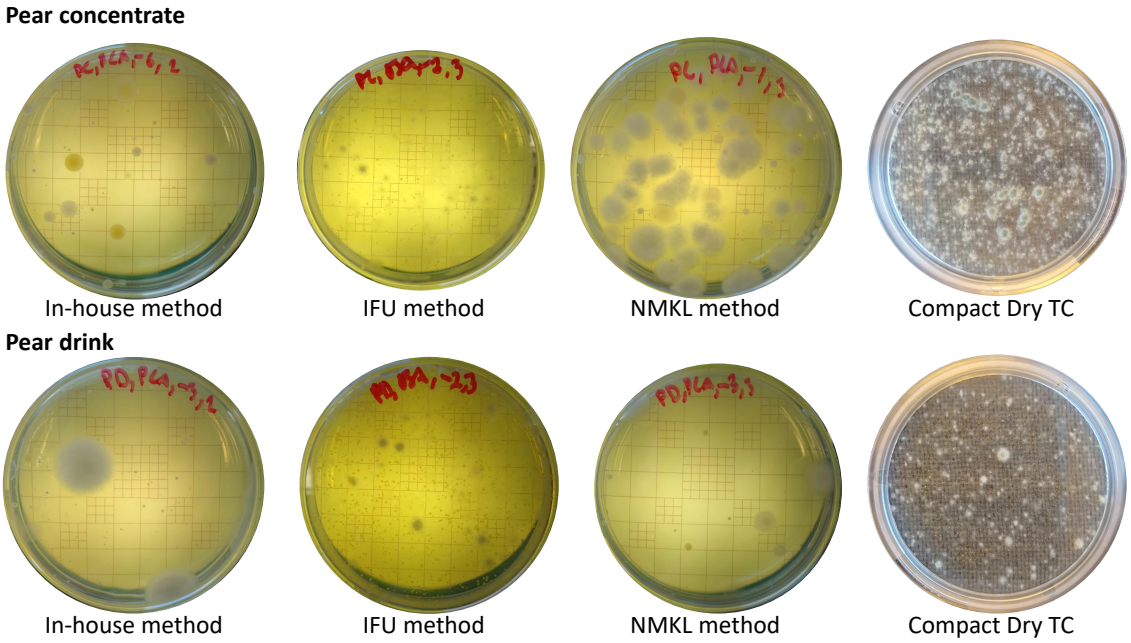

Figure 16 – In the picture above some of the petri dishes from the methods for aerobic mesophilic microorganisms (In-house method, NMKL method, IFU method and Compact Dry YMR) are shown. The upper four from the samples with pear concentrates, and the lower four from the the pear drink.

In table 19 the results for the plating with pear concentrate is shown. Both the in-house method and NMKL method showed a lacking correlation between the dilutions in the amounts of growth on the plates. For the first 2 dilutions there was too many colonies to count, but then between 20-100 CFU per plate for the rest of the agar plates. The moulds were however able to be counted, showing very similar results.

The Compact Dry TC showed over growth of mould on the first dilutions, but none on the other plates.

Table 19 – The results from the aerobic mesophilic microorganisms with the pear concentrate. The mean-value of CFU/mL are listed below. The in-house method and NMKL method showed similar results for the mould-count, but due to issues with showing correlations between the dilutiond of the samples and the colony-count were not possible to calculate. The – indicates no growth or no calculation possible. TNTC=Too numerous to count.

Method	Colonies [CFU/mL]	Mould [CFU/mL]
In-house method	–	$4.5 \cdot 10^2$
IFU method	$2.1 \cdot 10^6$	$1.6 \cdot 10^4$
NMKL method	–	$4.4 \cdot 10^2$
Compact Dry TC	$1.7 \cdot 10^4$	TNTC

In table 20 the results for the plating with pear drink is shown. Both the in-house method and NMKL method showed a lacking correlation between the dilutions and amounts of growth on the plates. The first 2 dilutions had too much growth to count, but then between 30-90 CFU per plate for the rest of the agar plates. The moulds were however able to be counted, showing very similar results. The Compact Dry TC showed over growth of mould on the first dilutions, but none on the other plates. The IFU method had too many colonies to count (> 300 CFU).

Table 20 – The results from the aerobic mesophilic microorganisms with the pear drink. The mean-value of CFU/mL are listed below. The in-house method and NMKL method showed similar results for the mould-count, but due to issues with showing a correlation between samples the colony-count were not possible to calculate. The – indicates no growth or no calculation possible. TNTC=Too numerous to count.

Method	Colonies [CFU/mL]	Mould [CFU/mL]
In-house method	–	$2.0 \cdot 10^3$
IFU method	TNTC	TNTC
NMKL method	–	$2.0 \cdot 10^3$
Compact Dry TC	$1.9 \cdot 10^4$	–

4.4 Further work

After this project, a plan of how the experimental work could be improved upon in several ways was developed. This can result in achieving more and better results to do statistical analyses with, making the results more comparable.

Specify strain and species: To achieve a higher repeatability of the results, specific strains of *Alicyclobacillus*, yeast and mould species could be used, instead of the mixed cultures used for this project. Either plated colonies from the companies own methods could be isolated and identified, or already typed strains of microorganisms could be bought and used.

Determining growth over time: Juice samples that should be used are those of interest for the company. For example, issues with *Alicyclobacillus spp.* has been seen with pear, orange and apple products, therefore these juices would be the first to be tested. One colony from a plate of the microorganism are added to the juice sample. After inoculation of the juice samples, the samples are mixed thoroughly and the samples are diluted and plated in triplicates according to each method. This will show how many CFU one colony is equal to. Lower dilutions of down to 10^{-8} or 10^{-9} should be made to avoid the issues with over-growth on the plates. At least 5 of these dilutions should be plated. The juice samples are then incubated at 25 °C for three days, with a new dilution series and plating is made each day.

This will show how many CFU are present before incubation after the inoculation and how the microorganisms grow during the incubation, allowing for determining how much the microorganisms can grow in each juice sample. If some samples inhibit growth due to factors like a high sugar content, the microorganisms will not grow during this incubation time. See figure 17 for proposed new work flow.

Increased amount of samples: An issue for this project, was too few dilutions plated, or to few sets of test. In the table 21 below a suggestion of the amounts agar plates for used for a set of triplicate of plating can be seen, as increasing number of dilutions that are plated. The addition of more than one juice sample is also listed. The dilutions of 10^{1} to 10^{-9} were assumed for the table, with 5-10 of the dilutions plated listed as "amounts of dilutions used".

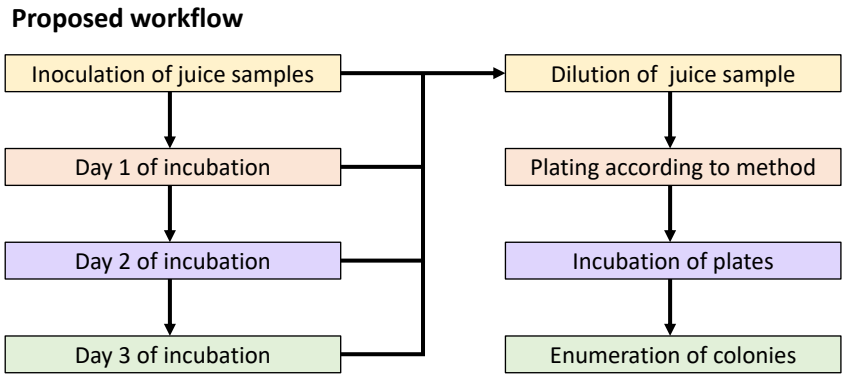

Figure 17 – A proposed new workflow, with additional plating done right after the inoculation of the juice samples.

Table 21 – Possibilities of amounts of agar plates used for a method. The amount is varied with different amounts of dilutions plates, as well as differing amounts of juice samples. Triplicates of each dilution is assumed.

Amount of dilutions used	**5**	**6**	**7**	**8**	**9**	**10**
1 juice sample	15	18	21	24	27	30
2 juice samples	30	36	42	48	54	60
3 juice samples	45	54	63	72	81	90

Pour plate method and surface plating: Since it was not a major focus of this project, the effect of surface plating or pour plating of the samples should be evaluated further.

For each dilution and juice sample plated, two sets of triplicates are made: Agar is melted and poured into 9 cm petri dishes and allowed to solidify, 0.1 mL of the samples are then spread on the surface of the agar. For the other samples 1 mL of the samples are added to a petri dish, then 15-20 mL of agar is added to the petri dish and mixed in a figure 8.

If the methods are equal, the plates should contain the same amount of CFU.

Confirmation of growth of ACB: According to the IFU method, growth of

Alicyclobacillus spp. can be confirmed by the following method:

Colonies from a petri dish with BAT agar and suspected growth of ACB are taken and carefully spread on the surface of a new petri dishes of BAT agar and PCA. The samples are incubated at 45 °C for 3 days. If the growth is *Alicyclobacillus spp.*, no growth should occur on the PCA due to the neutral pH (IFU, 2019).

This could be done for colonies on OSA plates from the in-house method as well, to confirm the selectivity of the method.

Additional methods: A method mentioned in the projects that was not used due to time constraints is the IFU method for yeasts and moulds. There are more methods and substrates that could be used for enumeration of ACB, mesophilic aerobic microorganisms and yeasts and moulds.

5 Discussion

5.1 *Alicyclobacillus spp.*

The three methods used, IFU method, in-house method and Veriflow method, have different pros and cons. The Veriflow method is very quick, but does not have the same potential for specificity as the cultivation based methods, without additional equipment. It is also comparatively expensive per test. It could however be used as a more quantitative test with additional equipment or with a standard curve of known concentrations of cells.

The IFU method using BAT as a substrate takes 5 days to complete, compared to the in-house method used now which pre-incubates the sample for 2 days after heat-treatment. In this regard the IFU method would be preferable. The shorter time is a huge advantage, if the methods are indeed comparable. This project failed to prove how and if the methods indeed are comparable, but with additional tests this could be shown. The IFU method also uses BAT agar, which have been shown to have a high recovery of *Alicyclobacillus* spores regardless if surface plating or pour plate method is used (Murray et al., 2007).

If the in-house method with OSA is used, it is recommended that the amount of sample spread on the surface is lowered from 1 mL to 0.1 mL. This will make enumeration of the colonies easier, as the sample will not have the same issues in absorbing into the substrate. The method of surface spreading is still recommended for the OSA since, according to previous work presented in the background, this results in a higher recovery of spores (Murray et al., 2007).

Both cultivation based methods use a heat-treatment of 80 °C to select for spores, since spores is the cause of issues in juice production. This is a method used by IFU and according to the method evaluation in (Murray et al., 2007). The difference between the heat-treatment for the two methods used are that the in-house method allows the juice sample to incubate at 45 °C for 2 days before plating. This

potentially results in plating of vegetative cells instead of spores. It would be recommended to evaluate if the 2-day incubation affect the results, and how much. If the differences are small, it could be removed.

The reasons for uncorrelated results on the plates for *Alicyclobacillus spp.* is not completely known. Cross-contamination between samples could be possible although this has been tried to be prevented with good hygiene. The incubator used did not hold the acquired 45 °C during the whole incubation period, but closer to 38 °C. This could explain the unknown green colonies present on the BAT and OSA plates. Re-contamination of the juice samples after the heat-treatment are also a possible reason for this.

5.2 Yeasts and moulds

All methods shows similar results, and the in-house and NMKL method is very similar in their execution. The incubation temperature is the same, 25 °C, and substrates are similar except for the antibiotic used. The in-house method has a shorter incubation time, due to the NMKL method having a incubation time of 5-7 days depending on if growth is shown after 5 days or not. The IFU method, not used in this project, shows promise in the shorter time of 3-5 days due to the slightly higher incubation temperature of 27 °C

The Compact Dry YMR plates have a color indicator which does not work with acidic samples such as fruit juices, but plates are fully readable without the color indicator. The samples could be adjusted to a neutral pH with a buffer, which can help the issues. The method is however very easy to use, with minimal preparation and steps. The incubation time is also short, only 3 days.

For detecting and analyzing samples for yeast and moulds, other methods than the ones used in this project exists, with variations of the ingredients of the substrates. The NMKL method for yeasts and moulds also recommends DRBC (dichloran rose bengal agar) for fruits and other fresh foods, and DG18 (dichloran glycerol agar) for foods with a water activity below 0.9 (NMKL, 2015). However many of these methods are similar to each other, and YCG, OGYE, DRBC and DG18 is very

widely used for enumeration of yeasts and moulds. Specific methods for identifying moulds like *Aspergillus spp.* that can produce aflatoxins exists, but this was not a focus of the project.

5.3 Aerobic mesophilic microorganisms

The plates showed different colonies of microorganisms, mould and probable yeasts as expected, as well as few colonies with other colors and shapes. Because of issues with over growth and correlation between dilutions, few comparable numbers exists. These issues with correlations was most prevalent with the in-house and NMKL method, and could be due to cross contamination. However, the count of moulds seems to be comparable over the different methods.

The IFU method and Compact Dry TC has the advantage of short incubation times of 2-3 days and 2 days respectively. The IFU method has the additional advantage of using OSA as a substrate, which promotes microorganisms that can grow on a lower pH that will pre present in the samples. For the Compact Dry TC, this is not the case, and the color indicator is not functional at the pH value of the samples. As with the Compact Dry YMR, the samples needs to be buffered for this.

The NMKL and in-house method uses the same substrate and incubation temperature, but the NMKL method has a shorter incubation time of 25 °C. The in-house method also showed growth on the plates after 3 days, indicating this incubation time is enough.

For aerobic mesophilic microorganisms, the choice of substrate is dependent on the samples that are analysed. PCA is good for a wide range of foods, but the substrate can be modified to be more compatible with samples with specific properties. In this way the growth of microorganisms present in the sample can be promoted (NMKL, 2013). Examples of this is the use of OSA for the IFU method, where the addition of orange serum promotes growth of microorganisms in juice and fruit samples (IFU, 1996). Another example is the addition of skim milk to PCA, this substrate is used for enumeration of mesophilic aerobic microorganisms in milk products (Merck, 2020b).

6 Future Work

During this work, several new paths were discovered. To obtain clearer results, an additional plating right after the inoculation of the juice, and for each day the juice samples incubate. This could be made to acquire information on how especially *Alicyclobacillus* grows in different kinds of juices during a period of 3 days. In addition to this, a larger sample size with more dilutions plated should be included.

Use of specific strains of *A. acidoterrestris* and typical yeasts and moulds that commonly spoil juice beverages could be used for methods in the future to ensure a higher specificity and repeatability.

The addition of confirmation of growth with the on BAT as potential *Alicyclobacillus spp.* could be done with the IFU method to further validate the method.

The effects of surface plating and the pour plate method should also be evaluated further, to be able to develop methods that work fast and precise for the Company. More methods, not included in this project, can also be evaluated.

Additional methods could also be tested, for example the IFU method for yeast and moulds.

7 Conclusion

The project presents several methods that can be used for identifying and quantifying *Alicyclobacillus spp.*, aerobic mesophilic microorganisms and yeasts and moulds. In table 22 an summary of the conclusions can be seen.

Table 22 – List of ingredients for OGYE agar (VWR, 2020)

	Recommended method	Motivation
Alicyclobacillus spp	Veriflow & IFU	No pre-incubation Fast primary results
Yeast & mould	In-house	Similar results Shorter incubation time
Aerobic mesophilic microorganisms	NMKL	Shorter incubation time

- The optimal pH range for *A. acidoterrestris* is 2.2-5.8 and temperature range of 42-53 °C, with a value of soluble solids of below 20 °Brix. *Alicyclobacillus* can grow in several fruit juices, orange, apple, pear and other citrus fruits The juices are spoiled by production of different compounds, mainly guaiacol.
- For *Alicyclobacillus spp.* the IFU method was deemed the most effective in terms of time and ability to recover the most of the samples with both practical use of the methods and literature as support. The Veriflow method is also a very effective way to quickly obtain initial information about potential spoilage.
- For yeast and moulds, the in-house method was recommended, due to similar results as the other methods, but a shorter incubation time than the NMKL method.
- For mesophilic aerobic microorganisms, since this saves 2 days, as well as gives similar results, the NMKL method is preferred over the other methods.

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A Appendix

Tagret	Method	Substrate	Time	Temperature	Surface spread/Pour plate method	Plate orientation
Alicyclobacillus spp.	IFU	BAT	5 days	45 °C	Pour plate	inverted
	In-house	OSA	2+5 days	45 °C	Surface spread	inverted
	Veriflow	-	3 hours	-	-	-
Yeast & Moulds	NMKL	OGYE	5-7 days	25 °C	Pour plate	upright
	IFU	OGYE	3-5 days	27 °C	Pour plate	upright
	In-house	YCG	5 days	25 °C	Surface spread	upright
	Compact Dry YMR	-	3 days	25 °C	-	upright
Aerobic mesophilic microorganisms	NMKL	PCA	3 days	30 °C	Pour plate	inverted
	IFU	OSA	2-3 days	25 °C	Pour plate	inverted
	In-house	PCA	5 days	25 °C	Pour plate	inverted
	Compact Dry TC	-	2 days	30 °C	-	inverted

Figure 18 – An overview of all methods used for this report, including their target organism, substrate, incubation time and temperature, if the method uses a surface spread och pour plate method and the orientation or the plate during incubation.