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Master Thesis

A Comparative Study Between Detection Methods for Spoilage Microorganisms in Fruit Juice

by

Lovisa Persson & Hannah Viklund

Division of Applied Microbiology
Lund University
Sweden
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Supervisors: Jenny Schelin, Stina Andrén, Rickard Blom

Examiner: Ed van Niel

Postal address
PO-Box 124
SE-221 00 Lund, Sweden

Visiting address
Naturvetarvägen 14

Web address
www.tmb.lth.se

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Abstract

The main concerns of food spoilage in fruit juices are the off-flavours and off-odours caused by microorganisms. In order to ensure high quality products, these microorganisms must therefore be eliminated or below a certain limit. Trials using pear concentrate, a ready-to-drink pear drink and a carbonated pear drink, were performed in order to compare two detection methods for three different groups of microorganisms: Total Count, Yeast & Moulds and *Alicyclobacillus* spp. The two methods compared were the standardised IFU methods for these three groups as well as the currently used In House (IH) method for the Company at whose disposal this study was conducted. The objective of the study was to investigate if a switch from the IH method to the IFU method could be carried out by the Company. In order to do this, the results between the methods need to be comparable and of similar sensitivity. The obtained results showed a tendency for similarity between the IFU and IH method for Total Count as well as Yeasts & Moulds. Regarding *Alicyclobacillus* spp., it seemed that the sensitivity of the method was higher for IH than IFU. The difference was in part suspected to be due to the pre-incubation step included in IH, not found in IFU. It is recommended that a pre-incubation period is added to the IFU method as allowed by IFU and that more trials are performed.

Populärvetenskaplig sammanfattning

Hur man bäst undviker att juicen smakar bacon

Har du någonsin öppnat en flaska juice på morgonen för att sedan upptäcka att den luktar bacon? I så fall är det möjligt att en typ av bakterie som kallas *Alicyclobacillus* (förkortad ACB) har börjat växa i juicen.

(Bestfotostudio, 2016)

Inom juice-industrin är ACB - tillsammans med jäst, mögel och andra bakterier som kan växa i sura miljöer - en av de främsta bovarna som gör att produkter måste slängas eller återkallas. Det är därför viktigt för företag att ha en bra metod för att hitta dessa typer av mikroorganismer tidigt i produktionen. Detta är relevant både ur ett ekonomiskt och miljömässigt perspektiv för att minska matavfall. Eftersom miljön i juice oftast är väldigt sur så är det inga farliga mikroorganismer som kan leva där, men det finns mikroorganismer anpassade till sura miljöer som kan göra så att juicen smakar eller luktar äckligt.

Detta arbete har jämfört två olika metoder för att hitta mikroorganismer i päronjuice för att se om metoderna kan jämföras med varandra. Den ena metoden är utvecklad av Företaget som arbetet utfördes på, medan den andra metoden är en standardiserad metod som används av många andra företag runt om i världen. Fördelen för Företaget att gå över till den standardiserade metoden är att de då lättare skulle kunna jämföra sina resultat med andra företag och producenter av exempelvis deras ingredienser.

Resultaten visar att de båda metoderna är jämförbara om det växer jäst och mögel eller bakterier såsom *Lactobacillus,* vilka tolererar sura miljöer, i juicen. Växer det däremot ACB i juicen så är de lättare att hitta med Företagets egen metod än med den standardiserade. Innan Företaget kan välja att byta metod bör de därför göra fler undersökningar med ACB.

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List of Abbreviations

ACB	*Alicyclobacillus* spp.
BAT	Bacillus acidoterrestris thermophilic (agar)
CD	Carbonated Ready-To-Drink Pear Drink (product sample)
CFU	Colony Forming Units
CO	Pear Concentrate (product sample)
IFU	the International Fruit and Vegetable Juice Association (method)
IH	the In House (method)
NB	Nutrient Broth
OGY	Oxytetracycline-Glucose-Yeast extract (agar)
OSA	Orange Serum Agar
PCA	Plate Count Agar
RT	Room Temperature
RTD	Ready-To-Drink Pear Drink (product sample)
TC	Total Count (microbiological method)
TFTC	Too Few To Count
TNTC	Too Numerous To Count
YGC	Yeast extract Glucose Chloramphenicol (agar)
YM	Yeast & Moulds (microbiological method)
YPD	Yeast extract Peptone Dextrose

1. Introduction

Food spoilage caused by microorganisms is a widespread problem throughout the food production sector and it has been estimated that approximately 25 % of the food produced globally is wasted due to microbial spoilage each year (Petruzzi et al., 2017). Because of the enormous losses this waste entails, both financially and from a sustainability perspective, food producers spend a great deal of money and time to try to reduce and prevent microbial food spoilage. Depending on the type of microorganism, the food product could receive an undesirable taste, texture and smell or, in worst case, even become a health hazard for the consumer (Salomão, 2018).

The fruit and vegetable juice industry is a section of the food market which has grown during the last decades and is forecasted to continue doing so as the population's interest in healthy food increases (Statista, 2022). Due to the low pH found in fruit and vegetable juices which makes it difficult for microorganisms to survive, these have traditionally not been subject to foodborne diseases, although some cases caused by microorganisms such as *Escherichia coli* and *Salmonella* have been reported. However, the greatest issues concerning food spoilage for fruit and vegetable juices are microorganisms which cause undesirable smell, taste and texture of the product (Salomão, 2018). In order to detect these spoilage microorganisms at an early stage, several different detection methods have been developed throughout the fruit and vegetable juice industry. These methods may differ in several ways such as the type of agar used, the incubation temperature and the incubation time.

When working with food quality in production it can be advantageous to use standardised methods as it will therefore be possible to compare one company's results with another or between the supplier of the raw material/ingredient and the producer of the final product. The Company with which this project is carried out has expressed an interest in switching from their own In House (IH) method to a more standardised one and previous projects have been carried out at the Company where different standardised methods have been compared with the IH method to determine which alternative might be viable to switch to (Lagerwall, 2020 and Bergsell, 2021). The International Fruit and Vegetable Juice Association (IFU) is an accredited

company which works with bringing forth standardised methods regarding food quality assurance, and was according to previous results the most suitable contender.

This project will therefore compare the methods provided by IFU with the Company's own In House methods to observe if it is viable to switch from the IH method to IFU's. It can be difficult for a company to switch between methods as there are several factors that must be taken into consideration before a potential switch can be made. Firstly, the two methods must produce similar results to ensure that the quality analysis performed by the Company will maintain a high standard. Secondly, a switch of methods might prove difficult as potential historical data from the old method could be lost as it will not be comparable to the new method. Finally, there are also logistical aspects that need to be considered such as availability of materials and the time it takes to implement the new method. It is therefore important that the two methods are thoroughly compared and investigated prior to a potential switch.

2. Aim of the Study

The aim of this project is to investigate if a switch from the Company's microbiological In House method to the International Fruit and Vegetable Juice Association (IFU method) could be carried out by the Company. As mentioned, the use of a standardised method would enable the Company to compare their results with the ones obtained by other companies or the supplier of raw materials. In order to implement a switch in detection methods, the results between the methods need to be comparable and of similar sensitivity. The In House methods used for three different groups of microorganisms, namely Total Count, Yeasts & Moulds and *Alicyclobacillus* spp., will be included in the study and compared with the corresponding method developed by IFU. The methods will be compared using three different pear drink samples. These will be: diluted pear concentrate (CO), ready-to-drink pear drink (RTD) and carbonated ready-to-drink pear drink (CD). To achieve a conclusive comparison the following objectives will be executed:

1. Perform the In House in parallel with the IFU method for Total Count in CO, RTD and CD and compare the obtained CFU/ml between the methods
2. Perform the In House in parallel with the IFU method for Yeasts & Moulds in parallel in CO, RTD and CD and compare the obtained CFU/ml between the methods
3. Perform the In House in parallel with the IFU method for *Alicyclobacillus* spp. in CO, RTD and CD and compare the obtained CFU/ml between the methods
4. Identify and discuss the challenges that comes with changing detection methods

A similar concentration of the obtained CFU/ml for the In House and the IFU method for the same microorganism group will be deemed as comparable and that the methods are of similar sensitivity.

3. Background

Common spoilage microorganisms in fruit and vegetable juices are different strains of yeasts and moulds, aerobic mesophilic microorganisms such as lactic acid bacteria and species of the spore-forming acidophilic bacteria called *Alicyclobacillus*. Although these three groups of microorganisms have been seen to affect a wide variety of fruit and vegetable juices such as citrus juice, tomato juice and pineapple juice, it seems as three of the most affected types of fruits are apple, pear and orange (Salomão, 2018).

Due to the Company's request, the type of fruit juice investigated in this study will be pear. Pear juice will therefore be further investigated below along with the three types of microorganisms mentioned above. As mentioned, three different variations of pear beverages will be used when comparing the methods: diluted pear concentrate (shortened CO) prior to processing, ready-to-drink pear drink (shortened RTD) and carbonised ready-to-drink pear drink (shortened CD). Furthermore, the detection method used in this study, namely cultivation will be investigated along with a rapid detection method currently used by the Company for the detection of *Alicyclobacillus* spp.

3.1 Spoilage microorganisms in fruit juice production

3.1.1 Aerobic mesophilic microorganisms

As fruit juices are high-acid foods with a pH below 4.2 (Tetra Pak Processing Systems AB, 2022), spoilage is generally caused by yeast and mould rather than bacteria (Adams et al., 2016).

One of the types of aerobic mesophilic microorganism, along with yeast and moulds, that are detected using the total plate count method (Methods & Materials: Total Count*)* is generally lactic acid bacteria (LAB) (Kregiel, 2015). The pH range for LAB growth differs between species. Some LAB species can survive and grow in pH as low as pH 3 because of their acid tolerance, however the growth optimum for many LAB species is at pH 6-7 (Schillinger et. al., 2006). Some possible metabolites of LAB are lactate, diacetyl, acetate, succinate and formate. These can contribute to a decrease in carbonation of the beverage as well as undesired changes in smell, taste and mouth-feel.

While less common than LAB, acetic acid bacteria (AAB) can also be found in fruit juice as they can grow in the pH range 3.0 - 3.8 (Kregiel, 2015). Furthermore, the growth temperature optimum for AAB is around 37 ℃ (Adams et al., 2016). Apart from producing similar metabolites as LAB such as lactate, acetate and succinate, AAB can also produce acetaldehyde, gluconate and ketones. These may result in undesired change in taste, haze and ropiness in the beverage (Kregiel, 2015).

3.1.2 Yeast & Moulds

Some of the most common moulds found in juice products are *Aspergillus*, *Fusarium* and *Penicillium* which can produce the mycotoxins aflatoxins, ochratoxin A, fusarium toxins and patulin that are harmful to humans (Kregiel, 2015). The optimum growth temperature for yeast is in the range of 25 - 30 °C. Some moulds are able to grow in refrigeration temperatures in the range of 0 - 5 °C, but most moulds have their optimum growth temperature in the range 25 - 35 °C (Bullerman, 2003).

There are however moulds that can grow at high temperatures and therefore also survive pasteurisation. These are called heat resistant moulds (HRM) and are a large concern for fruit juice producers. Their heat-tolerance is accredited to the formation of ascospores. The contamination of HRM can occur both from raw ingredients, processing environment and packaging (Rico-Munoz, 2017).

Yeast and moulds involved in food spoilage can grow over the wide range of pH 3 - 8. Their optimum is reported to be around pH 5 but some yeast and mould types are able to grow in pH < 2. In foods with high water activity, the yeast and mould compete with bacteria, but as the pH becomes lower, fewer bacterial species have the ability to grow which facilitates growth of yeast and moulds instead (Pitt & Hocking, 2022).

3.1.3 ACB

Alicyclobacillus spp. (ACB) is a thermoacidophilic, spore-forming bacteria that have the ability to survive pasteurisation processes applied in the juice industry. ACB grows in the temperature

range 20 - 70 °C. This means that only shelf-stable juices are at risk for spoilage by ACB as it cannot grow in frozen and refrigerated products. However, the added expenses of having a chilled storage and distribution of the product would be considerable. Juice products are generally pasteurised at temperatures between 80 - 100 °C. While ACB cannot grow during these conditions, they can survive as spores and germinate when conditions are more favourable (Molva & Baysal, 2015). It is hypothesised that their thermoresistance is related to the ω-cyclohexane fatty acids that are abundant in their cell membrane structure, which allows in dense packing of the fatty acids, leading to lower diffusion upon heating (Chang & Kang, 2004). In order to effectively kill ACB, the pasteurisation temperature needs to be above 100 °C (Tetra Pak Processing Systems AB, 2022).

The pH growth range for ACB is in the range pH 2.5 - 6.0 while the pH optimum for ACB is reported to be pH 4.5. However, as several bacterial species grow at pH 4.5, Chang & Kang suggested that the optimum isolation temperature for ACB would be pH 4.0 instead to be able to ensure specificity (Chang & Kang, 2005). The growth optimum temperature for a group of ACB species including *A. acidoterrestris*, which is one of the most common sources of ACB-caused spoilage, has been reported to be 40 - 55 °C (Pornpukdeewattana et. al., 2019). Growth of ACB is inhibited at a brix degree above 18 (Chang & Kang, 2004). In Figure 1 below, the rod-shaped morphology of *A. acidoterrestris* can be seen.

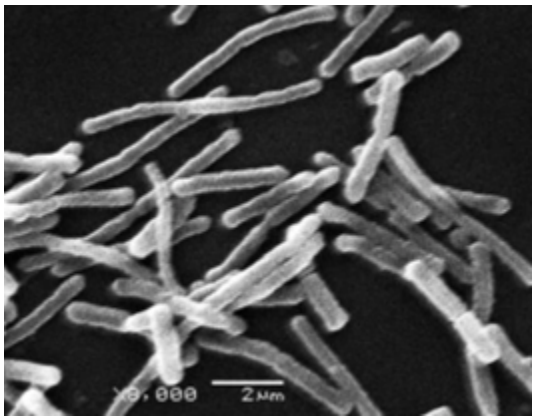


Figure 1. Image of Alicyclobacillus acidoterrestris *suspended in fruit juice using electron microscopy (Pei et. al., 2013).*

ACB is able to grow in both carbonated and non-carbonated drinks (Kregiel, 2015). However, according to Mermelstein, ACB requires access to oxygen to grow, suggesting that the growth in

carbonated beverages would be lower than in non-carbonated. Because of its aerobic characteristics, ACB growth is rarely seen for canned juices compared to glass- or or PET bottled juices with large headspace. PET is also an inadequate barrier for oxygen (Mermelstein, 2012).

While ACB is non-pathogenic, it is a quality concern because of its production of guaiacol and halophenols which creates an off-flavour and/or off-odour. The off-flavour caused by guaiacol has been described as smoky, likened to bacon crisps, as well as disinfectant-like (Chang & Kang, 2004). The perception of intensity of this smoky odour and flavour differs between individuals because of variations in a gene that codes for an odorant receptor to which guaiacol binds to. This difference in perceived intensity therefore affects the pleasantness of the flavour and people with a receptor that has a lower sensitivity to guaiacol have been found to find it more enjoyable (Mainland et. al., 2013).

A summary of all the growth and inhibition parameters for the three groups of microorganisms (aerobic mesophilic microorganisms, Yeasts & Moulds and ACB) discussed are presented in Table 1.

Table 1. Conclusive summary of the growth and thermal death requirements for aerobic mesophilic microorganisms, Yeasts & Moulds and ACB based on the above text. The thermal death temperature is based on the product being a high-acid product such as juice with a pH below 4.2.

	Aerobic mesophilic microorganisms	**Yeasts & Moulds**	**ACB**	**References**
Temperature growth range	10- 45 ℃ (Mesophiles)	0 - 60 °C (Moulds) Pasteurisation temperatures (HRM) 25 - 40 ℃ (Yeasts)	20 - 70 ℃	Mesophiles: Tetra Pak Processing Systems AB, 2022 Moulds: Bullerman, 2003 HRM: Rico-Munoz, 2017 Yeasts: Joseph & Bachhawat, 2014. ACB: Molva & Baysal, 2015
Optimum growth temperature	37 ℃	25 - 30 °C (Yeasts) 25 - 35 °C (Moulds)	40 - 55 ℃	Aerobic mesophilic microorganisms: Tetra Pak Processing Systems AB, 2022 Yeasts & Moulds: Bullerman, 2003 ACB: Pornpukdeewattana et. al., 2019
Thermal death temperature	< 95 ℃	95 ℃ 110-115°C (HRM)	> 100 ℃	LAB, Yeasts & Moulds, ACB: Tetra Pak Processing Systems AB, 2022
pH growth range	pH 3-7 (LAB)	pH 3 - 8 (Yeasts & Moulds)	2.5 - 6.0	LAB: Schillinger et. al., 2006

		pH < 2 (some yeast and moulds)		Yeasts & Moulds: Pitt & Hocking, 2022 ACB: Chang & Kang, 2005
Optimum pH for growth	pH 3.0 - 3.8 (AAB)	pH 5	pH 4.5 (for growth) pH 4.0 (for isolation purposes)	AAB: Kregiel, 2015 Yeasts & Moulds: ACB: Chang & Kang, 2005
Brix level for growth inhibition	-	> 50 °Bx	> 18 °Bx	Yeasts & Moulds: Lane Paixao dos Santos et al., 2020 ACB: Chang & Kang, 2004
Carbonation effects	Aerobic, but LAB and AAB can grow in carbonated drinks	Yeast primary contaminant in carbonated drinks but moulds can also grow	Aerobic, but can grow in both carbonated and non-carbonated drinks	LAB, AAB, Yeasts & Moulds, ACB: Kregiel, 2015
Ethanol percentage for growth inhibition	> 16 % (LAB)	> 5% (moulds) > 7-12% (yeasts)	> 5%	LAB: Calumba et al., 2021 Moulds: Dantigny et al., 2005 Yeasts: Tikka et. al., 2013 ACB: de Souza Sant'Ana et al., 2014

3.2 Pear beverage

In this project, three different types of pear juice products will be inoculated with microorganisms. The choice of pear was made due to the Company's request which was based on their own experience as well as available theory that marks pear as one of the most affected fruit juices by ACB. The ingredients and storage conditions of these pear drinks can be observed in Table 2 below.

Table 2. The name of the pear beverage products inoculated with microorganisms in the project and their abbreviations. A list of their ingredients as well as storage-conditions can also be observed.

Product	Ingredients	Storage
Pear concentrate (CO)	Pear concentrate with 70 Bx°	Ambient temperature, Silo tank outside.
Ready-to-drink pear drink (RTD)	Pear juice 14 % Sugar 7.5 Bx° Citric acid Pear aroma	Room temperature, 1.5 L aseptic cardboard packaging (Tetra Brik)
Carbonated ready-to-drink pear drink (CD)	Pear juice 14 % Sugar 7.5 Bx° Citric acid Pear aroma 5.0 g CO_2 / L	Room temperature, 330 mL Glass bottle

In order to determine the sugar content of a liquid, a Brix measurement can be performed which indicates the amount of sugar dissolved in the liquid. The soluble solid content of pear beverages has been measured in different studies to be between 15 - 70 °Bx, where 70 °Bx was reported for pear concentrate (Ibarz et al., 1987).

The Brix degree is an important tool when trying to estimate the risk of microbial growth in a food product. As some sugars can act as nutrients for microorganisms they are often able to promote microbial growth at lower concentrations. However, a too high concentration of sugar may instead inhibit growth and act as a preservative. This is mainly due to the osmotic effect where a higher solid content outside of the cells causes water to flow out of the microorganisms. Without enough water in the cells, the microorganisms cannot divide and the growth is thereby

inhibited (Mizzi et al., 2020). As the sugar content in a food product highly impacts the microbial growth it is of high importance that the producer is knowledgeable about the composition of their product.

The degree Brix at which growth of microorganism is inhibited varies depending on the microorganism in question and growth of ACB has as previously mentioned been observed to be inhibited at °Bx above 18 (Chang & Kang, 2004). Meanwhile, there are species of fungi such as *Neosartorya* spp. which have been proven to be able to grow at °Bx as high as 50 (Lane Paixao dos Santos et al. 2020).

As carbonated drinks contain CO_2, the amount of oxygen available for microorganisms is lower compared to a non-carbonated drink. This does not however mean that they are exempt from contamination. According to Kregiel, yeast is the primary contaminant in carbonated beverages as they are more tolerant against carbonation, but several other types of aciduric microorganisms such as different species of moulds, LAB, AAB and ACB are also able to spoil the product (Kregiel, 2015).

3.3 Detection methods

In order to ensure the quality of food products it is necessary to carry out microbiological examinations so that possible contaminants can be identified before the products are transported to the consumers (Adams et al., 2016). Two types of methods which are commonly used in routine microbiological analysis today are cultivation based methods and so-called rapid methods. The cultivation based methods will be used in this project and will therefore be investigated further along with the different types of agar used. However, since the Company also uses the rapid method Veriflow in their routine analysis, this will also be addressed below.

3.3.1 Cultivation based methods

Cultivation based methods promote growth of microorganisms into countable colonies by the use of a growth medium which might either be in liquid form or solidified with agar (Adams et al., 2016). The medium is formulated to promote growth of the microorganism in question whilst it may also inhibit unwanted microorganisms and it will thus vary depending on the purpose of the

study and the type of microorganism investigated (Salmonová & Bunesova, 2017). Since this study examines different types of microorganisms, different media are used and these can be observed in Table 3.

Table 3. List of media used in this study with their full name and abbreviation, their targeted microorganisms and the method(s) in which they are used. In House refers to the method used by the Company and IFU refers to the standardised method compared with the In House method in the project. ACB is an abbreviation for the bacterial species *Alicyclobacillus*.

Name of Medium	Aerobic Mesophilic Bacteria	Yeast & Moulds	ACB
OSA (Orange Serum Agar Medium)	IFU		In House
OGY (Oxytetracycline Glucose Yeast Extract Agar Medium)		IFU	
BAT (Bacillus Acidos Terrestris)			IFU
YGC (Yeast Extract Glucose Chloramphenicol Agar)		In House	
PCA (Plate Count Agar)	In House		

The type of cultivation method used in this study is an enumeration method more specifically known as the plate count method. This method enumerates the amount of microorganisms in a sample by allowing them to grow and form countable colonies on agar plates. The plate count method could be carried out in three different manners; by spread plate, pour plate or Miles and Misra drop plate. As the current In House methods as well as the methods developed by IFU use the pour plate method, this project will solely focus on this method. In the pour plate method, the sample is pipetted directly into the Petri dish and the molten agar is then poured into the Petri

dish, resulting in colonies growing in multiple layers instead of only on the surface of the agar plate (Adams et al., 2016).

In order to be able to count the number of colonies on the agar plates, it is often necessary to dilute the samples before they are added to the Petri dishes. It is widely accepted that the desired number of colonies on an agar plate should range between 30-300 and dilutions of the sample should therefore be carried out accordingly. The tenfold dilution series is most commonly used for this purpose where the sample is diluted tenfold with each dilution, and the desired dilutions can then be plated, often with a number of replicates to increase the confidence (Adams et al., 2016).

To calculate colony forming units (CFU) per ml, Equation 1 and 2 is used:

$$CFU/ml = \frac{No.\,of\ colonies \times Total\ Dilution\ Factor}{Vol.\,of\ culture\ plated\ in\ ml} \quad (1)$$

$$Total\ Dilution\ Factor = \frac{Final\ Volume}{Solute\ Volume} \quad (2)$$

Where *No. of colonies* are the number of CFU counted on the plate, *Total Dilution Factor* is the final volume of the sample divided by the solute volume and *Vol. of culture plated in ml* is the volume of diluted sample transferred to the Petri dish (Hogg, 2005).

3.3.2 Agar media

As there are several different types of agar media used in this project, the following section will give a short overview of each of the media. Tables 4 - 6 will give a comparative overview of the media used for each method whilst extensive comparative tables over the composition of the media can be found in Appendix: Agar Medium Compositions.

PCA (used for In House Total Count)

Plate Count Agar (PCA) is also called Standard Methods Agar and is commonly used for the enumeration of the total count of aerobic bacteria in food and dairy products as well as in water and wastewater samples (Vetbact, 2018). The medium was developed at the request of the American Public Health Association in 1953 by Buchbinder, Baris and Goldstein and the ingredients used today are the same as were suggested by Buchbinder et al. in 1953 (BD Diagnostic Systems, 2019).

PCA is a non-selective medium, meaning that it is free from selective supplements which might inhibit growth of certain microorganisms. As it is also rather rich in nutrients it is thereby a suitable medium for a total count enumeration (Neogen, 2022). The medium contains tryptone, which is a source of amino acids, and yeast extract, which contains peptides, sugars, minerals and B-vitamins. It also contains glucose, which is a source of carbon, water and agar which acts as the solidifying agent. Finally, the pH of PCA should be 7.0 (Vetbact, 2018).

OSA (used for In House ACB and IFU Total Count)

Orange Serum Agar (OSA) was developed in the 1950s by Hays when orange juice concentrate was contaminated with bacteria that could not grow on Lindegren's agar which his laboratory normally used (Hays, 1951).

The medium has pH 5.4 and is used for aciduric microorganisms such as LAB and ACB. Yeast that produces a buttermilk-like smell has also been found to grow on OSA plates. The buttermilk odour is suspected to be related to the production of diacetyl which LAB also produce. The medium contains tryptone as a source of carbon and nitrogen, while dextrose acts as a supplementary source of these elements. Phosphate supplies the medium with an osmotic buffer that facilitates cell survival of the microorganisms. The yeast extract provides the medium with Group B vitamins that contribute to growth of microorganisms. Lastly, the agar acts as the solidifying agent (Scharlau Microbiology, 2022).

A study was conducted by Witthuhn et. al., comparing five different media for their suitability of recovering *Alicyclobacillus* species from pear juice and white grape-juice. It was found that OSA

(pH 5.5), along with Potato Dextrose Agar (pH 3.7) had the highest recovery of vegetative cells. Moreover, OSA, after a heat treatment at 80 °C for 10 minutes, had the highest recovery of endospores (Witthuhn, Duvenage & Gouws, 2007).

Table 4. Ingredient list for PCA and OSA agar, used for the detection of Total Count. The PCA agar is used for the In House method whilst the OSA agar is used for the IFU method. A green box symbolises the ingredients presence in the medium whilst a red box symbolises that the ingredient in question is not present in the medium (Microbiologie Clinique, 2022) (Scharlau Microbiology, 2022).

Ingredients	PCA	OSA
Yeast extract		
Glucose anhydrous ($C_6H_{12}O_6$)/Dextrose		
Water		
Orange serum		
Tryptone		
Dipotassium phosphate K_2HPO_4		
Agar		

BAT (used for IFU ACB)

Bacillus acidoterrestris thermophilic (BAT) agar is specifically designed for *Alicyclobacillus acidoterrestris* (Avantor, 2022). It has pH 4.0 and is used in the IFU method no. 12 for detection and enumeration of thermophilic, acidophilic, spore-forming bacteria in juice products. BAT agar is composed of yeast extract and several salts which promotes growth of ACB, as can be seen in the Appendix (Scharlau Microbiology, 2022).

Murray et. al. performed a study in 2007, evaluating the performance of different agar and temperature for the growth and enumeration of various *Alicyclobacillus* species. Out of the media used, BAT were found to be one of the better alternatives compared to the others, including OSA agar (Murray et. al., 2007).

Witthuhn et. al. conducted a study in 2011, where three different methods for isolation of ACB species were compared. Inoculated peach juice samples were used on the three methods where the highest recovery of cells was found to be the IFU method no. 12 using BAT agar (Witthuhn et. al., 2011).

Table 5. Ingredient list for BAT and OSA agar, used for the detection of ACB. The OSA agar is used for the In House method whilst the BAT agar is used for the IFU method. A green box symbolises its presence in the medium whilst a red box symbolises that the ingredient in question is not present in the medium (IFU, 2019) (Scharlau Microbiology, 2022)

Ingredients	BAT	OSA
Yeast extract		
Glucose anhydrous ($C_6H_{12}O_6$)/Dextrose		
Calcium chloride dihydrate ($CaCl_2$ x 2 H_2O)		
Magnesium sulphate heptahydrate ($MgSO_4$ x 7 H_2O)		
Ammonium sulphate ($(NH_4)_2SO_4$)		
Potassium dihydrogen phosphate (KH_2PO_4)		
Zinc sulphate monohydrate ($ZnSO_4$ x H_2O)		
Copper sulphate pentahydrate ($CuSO_4$ x 5 H_2O)		
Manganese sulphate hydrate ($MnSO_4$ x 7 H_2O)		
Sodium molybdate dihydrate (Na_2MoO_4 x 2 H_2O)		
Water		
Orange serum		
Tryptone		
Dipotassium phosphate K_2HPO_4		
Agar		

YGC (used for In House Yeasts & Moulds)

Yeast extract glucose chloramphenicol (YGC) agar is a medium commonly used for the enumeration of yeast and moulds in primarily milk and dairy products, although it can also be used for analysis of water and other food products (Generon, 2022). The medium contains both yeast extract and glucose which acts as nutrients for the fungi whilst the agar acts as the solidifying agent. YGC also has a neutral pH of 6.6 ± 0.2 at 25 °C (Condalab, 2019).

Due to the presence of chloramphenicol, YGC is a selective medium. Chloramphenicol is a thermostable, broad spectrum antibiotic which inhibits the growth of bacteria and thus only promotes the growth of yeast and moulds. The antibiotic has a bacteriostatic activity and blocks the bacterial protein synthesis by binding to the bacterial 50S ribosomal subunit (Pubchem, 2022).

OGY (used for IFU Yeasts & Moulds)

Oxytetracycline-glucose-yeast extract (OGY) is a selective agar medium for the growth of yeast and mould. Mossel et. al. evaluated the specificity in 1969 by testing growth of *Bacillus* and *Enterobacteriaceae* on OGY. It was found, as previously observed, that the amount of oxytetracycline used in OGY inhibits growth of *Bacillus*. Limited growth of *Enterobacteriaceae* was observed, but further inhibition could be obtained through the addition of gentamicin (Mossel et. al., 1969).

Aside from the selective element in the medium (oxytetracycline), it also contains nutrients for microorganisms such as yeast extract and glucose as well as agar which acts as the solidifying agent (Corry et al., 1993).

Table 6. Ingredient list for YGC and OGY agar, used for the detection of Yeast & Moulds. The YGC agar is used for the In House method whilst the OGY agar is used for the IFU method. A green box symbolises its presence in the medium whilst a red box symbolises that the ingredient in question is not present in the medium (Sigma Aldrich, 2022) (Corry et. al., 1995) (Condalab, 2022).

Ingredients	YGC	OGY
Yeast extract		
Glucose anhydrous ($C_6H_{12}O_6$)/Dextrose		
Chloramphenicol		
Oxytetracycline		
Agar		
Water		

3.3.3 Rapid methods

Although cultivation based methods have several benefits such as them being inexpensive and relatively simple to use, there are also negative aspects that must be considered. For instance, they are time-consuming and laborious and it can therefore take a week or more before results are obtained. As not all microorganisms are culturable this could also mean that false-negative results are obtained (Woan-Fei Law et al., 2015). In an attempt to increase the speed and specificity of detection methods, so-called rapid methods such as nucleic acid-based methods have also been developed. Instead of cultivating the microorganisms in the sample, nucleic acids are used as the base for detection. The presence of specific microorganisms could for example be confirmed if a specific DNA sequence in the microorganism investigated was found in the sample (Salmonová & Bunesova, 2017).

However, even though rapid methods are faster, more specific and less laborious there still remain some challenges. For nucleic acid - based methods, there is always a risk that false-positive results are obtained as it is difficult to distinguish between viable and non-viable cells. Furthermore, the presence of PCR inhibitors can also affect the results by inhibiting PCR which may result in a false-negative result (Woan-Fei Law et al., 2015).

The Company currently uses a PCR-based method called Veriflow in their routine analysis. The Veriflow method begins by amplification of the target DNA, which in this case is a specific DNA sequence for ACB. In the amplification, the target DNA will be copied according to the three cycles of PCR: denaturation, primer annealing and extension. In the denaturation step, the target DNA strand will first be separated into two single strands and primers will then hybridise to the target DNA in the primer annealing step. The last step in the amplification is the polymerisation process where the primers are extended along the single-stranded DNA templates until two identical, double-stranded DNA chains have been created. This process is repeated over thirty times, thus resulting in an amplification of the target DNA. As false-negative results due to PCR inhibitors are a common issue for rapid methods, the Veriflow method uses inhibitor - resistant DNA polymerases. After amplification, the amplicon (the amplified target DNA) is transferred to a test cassette. The amplicon flows through a set of capillaries onto the test line which is immobilised with captured antibodies and is then trapped between the antibodies and a hydrated gold-protein conjugate. The amplicon aggregates on the test line which results in a visible line on the cassette. There is also a control line on the cassette which means that a negative result has one line whilst a positive result has two lines and this can be observed in Figure 2 (Invisible Sentinel, 2020).

Figure 2. Veriflow test cassette for the detection of ACB. Presence of one line is equal to a negative result whilst presence of two lines is equal to a positive result (Invisible Sentinel, 2022).

The risk of false-positive and false-negative results is of great concern for food producers and something which they strive to prevent as much as possible. From an economic standpoint a false-positive is an issue as the product will be retained from the market or, as in the case of the Company, it will be treated at a higher temperature to ensure safety. This is obviously more

energy demanding and thus more expensive. However, as a false-negative result could result in a contaminated product being brought out to the consumer this is perhaps of even greater concern than false-positive results. Not only would it damage the reputation of the food producer, but depending on the type of microorganism present in the product it could also become a direct health hazard to the consumer. In order to try to prevent false results as much as possible it is therefore common to use rapid detection methods for routine analysis but include cultivation based methods as a complement.

4. Methods & Materials

In 4.1 Materials, the used media in the study are listed and when applicable, the methods used to prepare the media are also presented. In 4.2 Experimental Design, the pre-trial and detection methods are described.

4.1 Materials

A comprehensive list of the used materials is presented in Appendix: Materials. In Table 7 below, the agar media and other solutions used are listed.

Table 7. A list of the agar media and growth media used in the project, their manufacturer and their ingredients. PCA was used for the In House Total Count Method, YGC for the In House Yeast & Moulds method, OSA for the In House ACB method as well as the IFU Total Count method, OGY for the IFU Yeast & Moulds method and BAT for the IFU ACB method. Nutrient broth was used for the mixed cultures and YPD was used for the Yeast & Moulds mixed culture as a nutrient source. NaCl was used to dilute the samples prior to plating.

Product	Manufacturer	Ingredients
Plate count agar (PCA)	VWR chemicals	See Table 45
Yeast glucose chloramphenicol agar (YGC)	VWR chemicals	See Table 46
Orange serum agar (OSA)	VWR chemicals	See Table 45
Oxytetracycline Glucose Yeast-extract Agar (OGY)	Oxoid	See Table 46
Bacillus AcidoTerrestris Agar (BAT)	Döhler	See Table 44
Nutrient broth	VWR chemicals (the Company) Merck (LTH)	8 g Nutrient broth 1000 g distilled water (autoclaved for 15 min at 121 °C)
Yeast extract peptone dextrose (YPD)	Merck	10 g Peptone 5 g Yeast Extract 500 ml distilled H_2O 10 g glucose (autoclaved for 15 min at 121°C)
NaCl 0.9 %	Merck	9 g NaCl 1000 g distilled water (autoclaved for 15 min at 121 °C)

4.1.1. Preparation of OGY medium

The OGY agar was prepared by suspending 18.5 g of the agar blend powder (Oxoid) in 500 ml of distilled water. The medium was mixed. According to the manufacturer's instructions for preparation of the OGY agar, the medium is to be autoclaved at 115 ℃ for 10 minutes. However, the autoclave available for usage was not adjusted for this precise setting and 121 °C for 10 minutes was applied instead as it was deemed that this deviation would not affect the quality or composition of the agar medium. According to the manual, the autoclaved agar mixture should be cooled to 50 °C, at which the OGY selective supplement should be aseptically added to the agar mixture. The agar should then be mixed and poured into plates to be used for the spread plate method. However, as the IFU method (no. 3) advocates the pour plate method, another deviation to the OGY preparation instruction was made. The agar mixture was cooled to solidification and when it was to be used in the IFU method for Yeast & Moulds, the agar mixture was melted, cooled to 45 °C in a water bath (OneMed Water Bath TECHNE TE-10A Tempette®) after which the selective supplement was aseptically added. The agar mixture was mixed by swirling the bottle and then immediately used for pour plating according to the IFU method no. 3.

4.1.2. Preparation of YPD medium

Peptone and yeast extract were dissolved in 400 ml distilled water. The solution was autoclaved at 121 °C for 15 minutes. Glucose was dissolved in 100 ml of distilled water. The solution was sterilised with a 0.22 µm filter and the two solutions were mixed together.

4.2 Experimental design

The experimental design included preparation of the mixed culture used to inoculate the products in order to perform and compare the two methods. A pre-trial in order to determine the inoculation volume of aforementioned culture media was also conducted. Additionally, preparation of the Company's products used in the study was made. The pear concentrate (CO) and ready-to-drink pear drink (RTD) were retrieved from the production line, while the carbonated pear-drink (CD) was made in the product development department using RTD. The method used to compare the IFU and IH method is described in detail in section 4.2.4. Two

independent, biological trials were performed for each method (TC, YM and ACB). These trials will be referred to as Trial 1 and Trial 2 later in the Method as well as Results. For each trial, two replicates of the same sample were plated. These are referred to as Replicate 1 (R1) and Replicate 2 (R2).

4.2.1 Growth of starter cultures

Mixed starter culture to inoculate the products were made using the Company's own microorganisms to simulate a realistic case of contaminated products. The microorganisms were obtained from either agar plates or the Company's products known to have a high degree of contamination. These were provided by the QC lab. The process to achieve this live mixed culture is divided into two sections: Yeast & Moulds and Total Count as one section and Growth of ACB cultures as the other.

Yeast & Moulds and Total Count

Colonies from agar plates with either Yeast & Moulds (YM) or Total Count (TC) were transferred to 100 ml sterile beakers filled with either 100 ml Nutrient Broth or 100 ml RTD. The samples were then left in the incubator at 30℃ for 4 days. As the growth rate for these samples were slow, new cultures were prepared using the old ones. The new samples were made using 20 ml RTD, YPD (Yeast extract peptone dextrose, Merck) and freshly prepared Nutrient Broth (NB, VWR & Merck). These were inoculated with 0.4 ml from the NB and RTD samples made at the Company earlier. For the TC-samples, two agar plates with colonies were also used to inoculate samples. For the YM-samples, only the 100 ml samples prepared at the Company earlier were used because of their inability to grow on agar plates.

The samples were vortexed and placed in an incubator (New Brunswick Scientific, innova 40) set at 30 ℃ and 150 rpm. The lids of the samples were slightly opened to allow air in. The samples were tilted to create a greater surface area for oxygen transmission. The samples were left in the incubator overnight and then aired by opening the lid and closing it again as well as vortexed. They were placed back in the incubator for another 2 days. The OD of the samples were measured and the results are presented in the Appendix: Preparation of mixed stature culture media for TC and YM. The chosen samples to continue to use as inoculation samples

were YM-RTD-YPD (YM inoculated in RTD and re-inoculated in YPD) and TC-RTD-NB (TC inoculated in RTD and re-inoculated in NB) respectively as they had the highest OD values.

The cultures were renewed every other week to keep the mixed culture alive. This was done by transferring some of the current mixed culture to a volume of fresh NB (for TC) or YPD (for YM). Dates of when the media were renewed are presented in Tables 10 and 11 in Results.

Growth of ACB cultures

The starter cultures of ACB were made in a similar way to those of YM and TC. Sterile falcon tubes (50 ml) were filled with 20 ml of four different media: NB (VWR Chemicals, prepared at the Company), NB (Merck, prepared at LTH), pH-adjusted NB (Merck, prepared at LTH) and ready-to-drink pear drink. The pH-adjusted Nutrient Broth was made using about 0.5 g of pulverised citric acid and dissolving it into the NB until pH 3.5 was reached. This pH was chosen because the pH of the NB was measured to be around pH 6.7 while the RTD was around pH 3.1.

The filled falcon tubes were either inoculated using 0.4 ml of a water sample containing ACB, which was provided by the QC lab at the Company or colonies taken from agar plates with growth from this ACB water. The inoculated samples were vortexed and placed in an incubator (LabRum Klimat) at 45 ℃. After 1 day they were opened to let air in and vortexed. They were returned to the incubator. This step was repeated every weekday.

A trial using the ACB culture with the highest OD measured (ACB-water-NB-LTH-pH) was conducted according to both IFU and IH method. As few CFU were observed, a new ACB culture was made using the IFU RTD sample from the trial as it had the highest number of colonies. 2 ml of IFU RTD was mixed with 18 ml NB. The medium was incubated at 45 ℃ and the ACB culture medium was renewed every other week with fresh NB to provide new nutrients to the microorganisms in the same way as done for the TC and YM cultures.

4.2.2. Inoculation sample size pre-trial

A pre-trial to decide the inoculation volume of mixed culture was conducted using the YM sample with the highest OD (YM-RTD-YPD). Two volumes were investigated: 1 ml and 100 µl.

These volumes were inoculated into 100 ml of RTD. The IH method for YM was followed (The Company, 2022). Based on the results, an inoculation volume of 100 µl was chosen for future trials.

4.2.3. Preparation of product samples

As mentioned in section 2.2, the three products used in this experiment are pear concentrate, RTD and carbonated pear drink. Originally, a pear cider was proposed as the carbonated pear drink, but due to it containing the preservative E202, it was discarded as it would inhibit the growth of the inoculated microorganisms. As a carbonated pear drink was still desirable by the Company to include in the experimental design, the RTD was carbonated instead. The RTD was carbonated by the use of Steinfurth LCS710P Laboratory Carbonation System, and the carbonation level was set at 5 g/L. After carbonation, the bottles were sealed with a cap (super Grifo TCSG capper) and then placed in a pot on the stove for pasteurisation. The pot was filled with water to a level which rose above the liquid level in the bottles. The water was then heated to 72 °C. When the desired temperature had been reached, the pot was removed from the stove and the bottles were gently tipped so the top of the bottles would also be pasteurised.

The pear concentrate was collected from a tank located outside at the Company, and in order to avoid contamination the concentrate was filled into 200 mL glass flasks which had previously been autoclaved at 140 °C.

Prior to inoculation, the brix of the pear concentrate was measured to be 68 Bx° (Bellingham + Stanley™ RFM340+ Refractometer). After a discussion with the QC personnel, 16 g of the pear concentrate was therefore used to obtain a 100 ml dilution of 11.5 Bx°. The pear concentrate was diluted using sterile distilled water.

4.2.4. Comparison between the IFU - and the IH method for TC and YM

The general set-up for the comparison between the IFU - and IH method for Total Count (TC) and Yeast & Moulds (YM) are similar. Sterile 120 ml beakers were filled with 100 ml of either diluted CO to around 11.5 Bx°, CD or RTD. One sterile beaker was filled for each product to a total of three samples. This ensured that the same sample was used for the IH and the IFU

method respectively. The OD of the culture medium was measured (Spectrophotometer DR 2800) and noted. The 100 ml product samples were inoculated with 100 µl of mixed culture.

After inoculation, zero-samples i.e., agar plates made with samples from day 0, were made for both the IFU and IH method. A tenfold serial dilution series was made using sterile 0.9 % NaCl for the inoculated samples. The dilutions to be plated were chosen based on the expected growth observed in previous trials (data not shown) and on the measured OD-value. A volume of 1 ml of sample from the chosen dilutions were added to marked Petri dishes by pipetting with sterile straw tips (VWR). One plate for the used 0.9% NaCl solution was also made as a control.

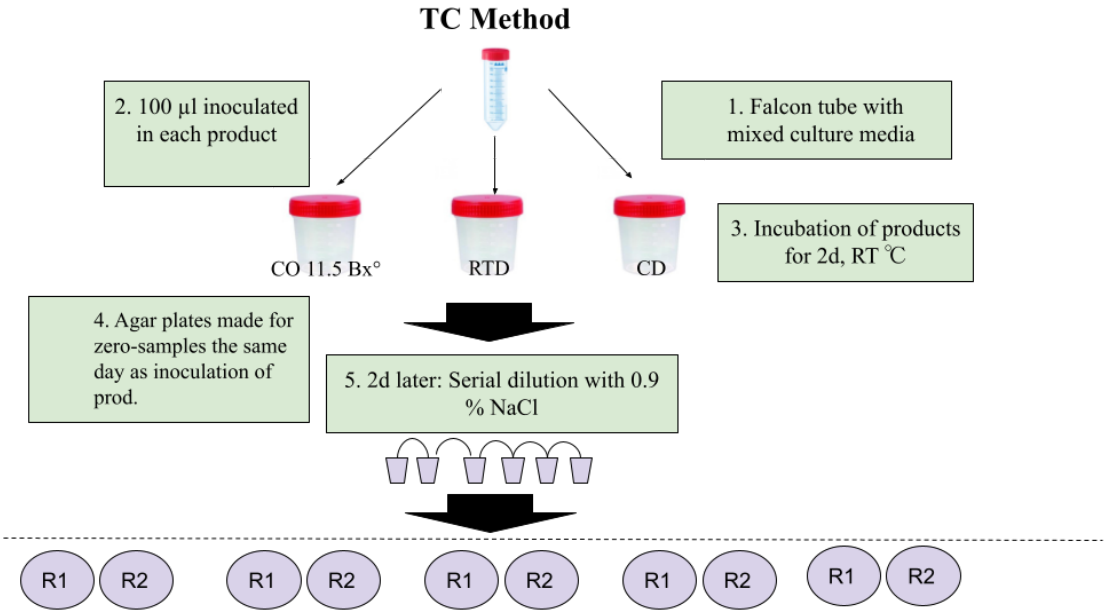
The time to melt the agar depended on agar type; for BAT and OGY it took around one hour while the other types only needed 25 minutes. When the agar had melted, the bottles were moved to cool down in a water bath set at 45 °C. The agar medium had cooled down to 45 °C after 25 minutes. About 15 ml of agar was poured into the prepared Petri dishes filled with 1 ml of sample. The control plate for the agar medium was poured first to avoid contamination. The plates were gently rotated on the table in circular motions to mix the agar with the sample. The plates were left to set on the workbench. Once set, they were placed in an autoclave bag and into its incubator.

The incubation time and temperature differed between the methods. Once the respective incubation time had been reached, the plates were taken out to be counted using a colony counter (Dr. N. Gerber Original), equipped with a marking pen. A counting pen (VWR) was used to facilitate the counting of CFU. Photographs were taken of all plates for record keeping.

After 2 days of incubation for TC and 3 days for YM, a new tenfold serial dilution series was made. The pour plate method was executed as described above in the same way as for the zero-sample plates. In the sections below, more details for the total count, yeast and mould and ACB method are given.

In Figure 3 below an overview of the experimental designs for the TC trials can be viewed. The difference between the IH and IFU method is also highlighted by the use of red boxes in the

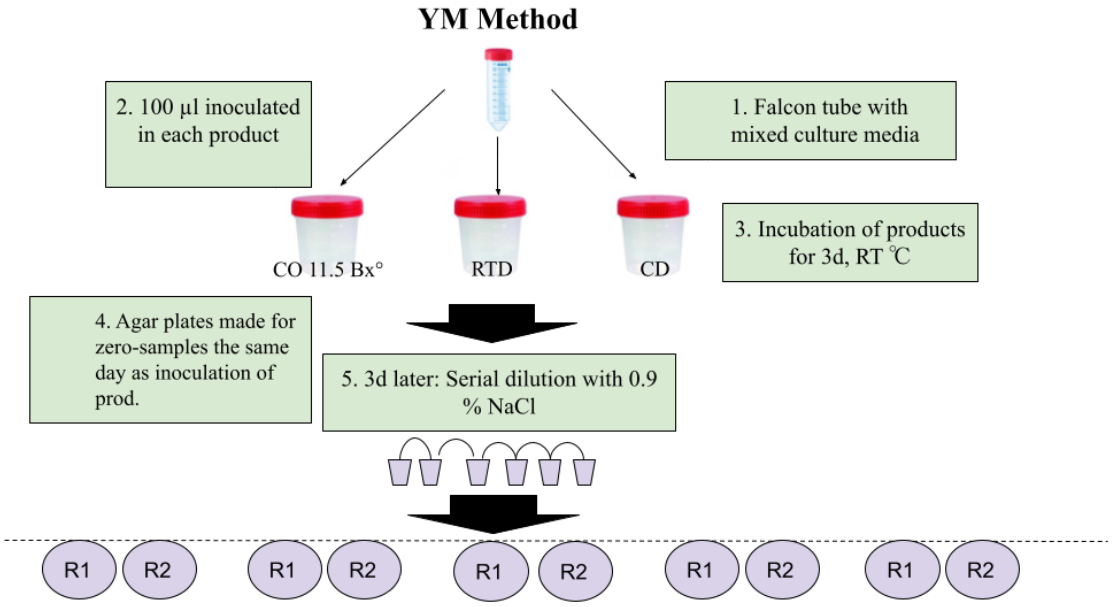
figures. The deviation in the method starts after the dotted line in the figure. The main difference in the TC method between IH and IFU was the agar sort used, incubation time and incubation temperature. The incubation time in the IFU method (IFU Microbiology Method no. 2) was updated in October 2022 from 2-3 to 3 days (IFU, 2022).



Differences in method		
	IH	**IFU**
Agar	PCA	OSA
Incubation temperature	25 ℃	30 ℃
Incubation time	5 d	3 d

Figure 3. Overview of the method used for TC for both IFU and IH. CO is the abbreviation for pear concentrate RTD for ready-to-drink pear drink and CD is for carbonated ready-to-drink pear drink. R1 stands for the first replicate and R2 for the second replicate of the plates made for the same sample. Green boxes indicate that the method step was the same for both IFU and IH. After the dotted line the method started to differ between IFU and IH as indicated by the red boxes below the dotted line. The difference in the TC method was the medium used (OSA for IFU, PCA for IH), the incubation time (3 days for IFU and 5 days for IH) as well as the incubation temperature(30 °C for IFU, 25 °C for IH).

The YM plates for both the IH method for YM were incubated right-side up. According to the IFU method no. 3, the plates are to be incubated right-side up at 27 ± 2 °C for 3-5 days (IFU, 1996). However because of a shortage of heating cabinets, the plates were incubated at 30 °C for 3 days. An overview of the experimental setup for the YM method is shown in Figure 4, where the differences between the IFU and IH method is highlighted by the use of the red boxes. As with the TC method, the difference in the YM method was the agar sorts used, the incubation time and incubation temperature.



Differences in method		
	IH	**IFU**
Agar	YGC	OGY
Incubation temperature	25 ℃	30 ℃
Incubation time	5 d	3 d

Figure 4. Overview of the method used for YM for both IFU and IH. CO is the abbreviation for pear concentrate RTD for ready-to-drink pear drink and CD is for carbonated ready-to-drink pear drink. R1 stands for the first replicate and R2 for the second replicate of the plates made for the same sample. Green boxes indicate that the method step was the same for both IFU and IH. After the dotted line the method started to differ between IFU and IH as indicated by the red boxes below the dotted line. The difference in the YM method was the medium used (OGY for IFU, YGC for IH), the incubation time (3 days for IFU and 5 days for IH) as well as the incubation temperature (30 °C for IFU, 25 °C for IH).

4.2.5. Comparison between the IFU - and the IH method for ACB

As seen in the flowchart in Figure 5, the experimental setup used to compare the ACB IH - and IFU method is almost identical to the ones used for TC and YM but with a few exceptions. A sample volume of 100 ml was still used, but the samples were placed in autoclaved 250 ml glass bottles instead of the sterile plastic beakers. This was because of a heat treatment applied to the samples that is included in the methods. Additionally, six samples were prepared instead of three as the IFU and IH heat treatment differs from each other.

The measured OD-values for ACB on the day of inoculation were lower than the ones obtained for TC and YM and thus an inoculation volume of 1 ml for trial 1 and 2 ml for trial 2 was used instead of 100 µl. Moreover, lower dilutions were chosen for plating (generally undiluted to 10^{-2}) as the OD for the ACB culture was lower than for TC and YM.

Another difference for the ACB method compared to the other methods, is the included heat treatment of the product samples after the products have been incubated at room temperature for 2 days. The IFU and IH samples were heat treated separately as the methods used different temperatures and times. The IFU samples were placed in a water bath along with an additional glass bottle filled with 100 ml of water. In this bottle, a thermometer (ALLA France) was placed. When the temperature of the water in the bottle reached 80 °C, the temperature was held there for 10 minutes (IFU, 2019). The IFU samples were removed from the water bath and left to cool at the workbench to room temperature. The temperature of the water bath was adjusted and the IH samples were placed in it. When the temperature of the water-contained bottle reached 82 °C, this temperature was kept for 15 minutes. The IH samples were removed from the bath and cooled on the workbench for a few minutes. The IH samples were then placed in the 45 °C heating cabinet for 2 days of pre-incubation (The Company, 2022).

Once the IFU samples had cooled to room temperature, a tenfold dilution series using sterile 0.9% NaCl was made. 1 ml of sample from the chosen dilutions were added to the Petri dishes by pipetting with sterile straw tips (VWR). One plate for the used 0.9% NaCl solution was also made as a control. BAT agar was melted and then cooled to 45 °C. About 15 ml of the BAT agar was poured into marked plates, starting with the empty plate used as control for the agar

medium. After the plates were set, they were inverted, placed in an autoclave bag and incubated bottom-side up at 45 ℃ for 5 days (IFU, 2019).

Two days later, the IH samples were removed from the heating cabinet. Tenfold dilution series were made. A volume of 1 ml of the sample from the chosen dilutions were transferred to Petri dishes. The OSA agar was melted and cooled to 45 ℃. About 15 ml of the OSA agar was poured into the prepared plates, starting with the empty plate used as control for the agar medium. After the plates were set, they were placed in an autoclave bag and incubated right-side up at 45 ℃ for 5 days (The Company. 2022).

When the respective incubation times for the plates were reached, the plates were counted, noted and photographed.

The main differences between the two methods are the agar sort used, the way the plates are incubated (bottom-side up vs. right-side up), the heat treatment temperature and time as well as the inclusion of a pre-incubation in the IH method. An overview of the method is presented in Figure 5 where these differences are highlighted by the use of red boxes for the steps where the methods deviate from each other and green boxes for steps that are the same for both methods.

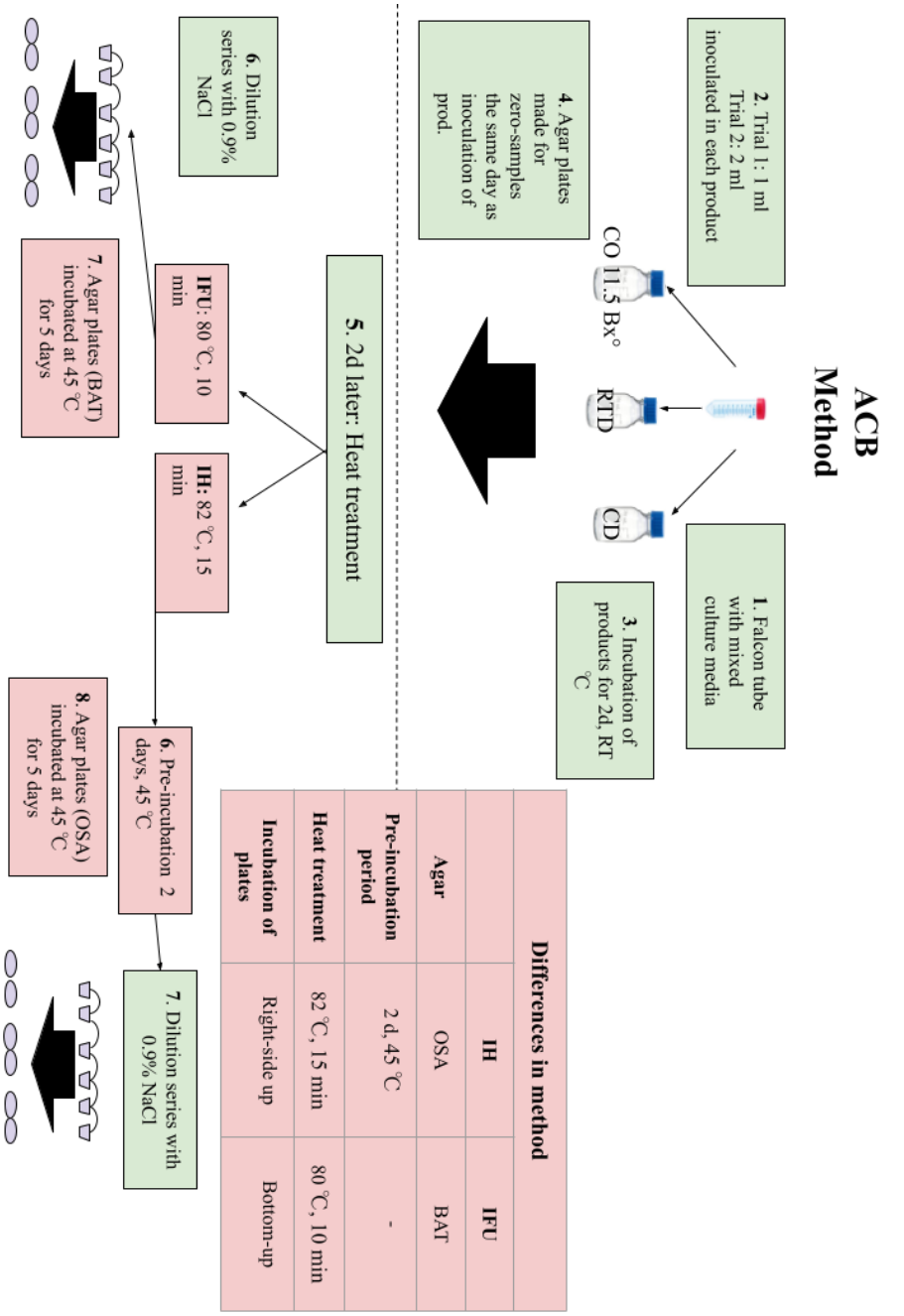


Figure 5. Overview of the method used for ACB for both IFU and IH. CO is the abbreviation for pear concentrate RTD for ready-to-drink pear drink and CD is for carbonated ready-to-drink pear drink. Green boxes indicate that the method step was the same for both IFU and IH. After the dotted line the method started to differ between IFU and IH as indicated by the red boxes below the dotted line. The difference in the ACB method was the medium used (BAT for IFU, OSA for IH), the heat treatment (80 °C, 10 min for IFU and 82 °C. 15 min for IH) as well as the pre-incubation after the heat treatment included in the IH method (2 days, 45 °C).

ACB method comparison on a real sample

The IFU- and IH method were also compared using a sample of orange juice concentrate that the lab had detected ACB growth in previously. The sample was taken from the freezer and thawed. The IH method was followed in preparing the sample. 30 g of the thawed concentrate was placed in a 250 ml sterile, glass bottle. 200 ml of sterile distilled water was added to the concentrate. The sample was mixed. In total, two samples were prepared: one for the IH method and the other for the IFU method.

The two bottles were heat treated according to the same method as described for the simulated ACB samples above and shown in Figure 5. After this step, the method was identical to the method described for ACB before (3.2.4. Comparison between the IFU - and the IH method: ACB).

5. Results

In the following section the results from the experiments will be presented. As mentioned in the Method, two independent, biological trials were performed for each method (TC, YM and ACB). These trials are referred to as Trial 1 and Trial 2. For each trial, two replicates of the same sample were plated. These are referred to as Replicate 1 (R1) and Replicate 2 (R2).

There were several experiments carried out prior to the trials reported for TC, YM and ACB below. However, these experiments had multiple challenges such as contamination of material and are thus not reported in this report. These previous experiments did however affect the decision in which dilutions to plate for Trial 1 and aided the development of the experimental setup.

5.1 Pre-trial

The results from the pre-trial on inoculation volume can be viewed in Appendix: Pre-trial. The obtained concentration for the RTD sample inoculated with 1 ml of mixed Yeast & Mould culture had a similar concentration (1×10^9 CFU/ml) to the RTD sample inoculated with 100 µl (3×10^9 CFU/ml). However, CFU in the interval 30-300 was observed on plates with a lower dilution (10^{-4}) for the 100 µl samples compared to the 1 ml samples which had CFU in the interval for dilution 10^{-5}. As lower dilutions are more time and material efficient, an inoculation volume of 100 µl was chosen for the continuation of the experiments as they were deemed most suitable.

5.2 Brix

The pear concentrate was diluted to a suitable brix degree before being inoculated with microorganisms to ensure growth could occur. A brix degree between 11-12 was chosen as a suitable degree in conversation with employees at the Company, and the exact brix degree for the diluted pear concentrate for the different samples can therefore be observed in Table 8.

Table 8. Measured brix degrees using Brix refractometer (Bellingham + Stanley™ RFM340+ Refractometer). The brix degree was measured when diluting the pear concentrate using sterile water prior to inoculation.

Sample	Measured °Bx Trial 1	Measured °Bx Trial 2
ACB-IH CO	11.51	10.68
ACB-IFU CO	11.47	11.35
YM CO	11.47	11.57
TC CO	11.13	11.69

5.3 pH

The pH-values of the used products (Pear concentrate, Ready-to-drink pear drink and Carbonated pear drink) in this study can be observed in Table 9 along with the pH-values of the different nutrient broths prepared.

Table 9. Values of measured pH in the used products (Pear concentrate, Ready-to-drink pear drink and Carbonated pear drink) as well as nutrient broths. The pear concentrate was measured in its undiluted form and the diluted pear concentrate used for inoculation could therefore be estimated to be higher. The Nutrient Broth used for the microorganism mixed cultures was Nutrient broth autoclaved at LTH (Merck). The two other nutrients broths listed in the table were used for making the mixed cultures at the start of the study but the one made at the Company (VWR Chemicals) were deemed not to be autoclaved in a correct manner and therefore discarded, while the pH-adjusted broth was used in the initial mixed culture medium made for ACB.

Sample	Measured pH
Undiluted pear concentrate	3.91
Ready to-drink pear drink	3.13
Carbonated ready to-drink pear drink	3.06
Nutrient broth (LTH, Merck)	6.73
Nutrient broth (LTH, pH-adjusted, Merck)	3.50
Nutrient broth (the Company, VWR Chemicals)	7.26

5.4 Optical Density

In order to get an idea of the starting concentration of microorganisms in the samples, and thereby also how high dilutions might be necessary to plate, the OD-values of the original microorganism cultures were measured and these can be observed in Tables 10 - 12 below.

Table 10. Measured OD-values for Total Count (TC) samples, cultured first in ready-to-drink pear drink and then transferred to nutrient broth (NB prepared at LTH, Merck). Measurements were taken prior to inoculation into product samples as well as during the development of the medium enrichment. The presented OD-value is the one measured prior to inoculation. The age of the medium differed between the trials and is also presented.

Sample	Trial	Date	Medium renewed	Medium age since renewal [days]	OD-value
TC-pear-NB	1	2/11	13/10	20	1.49
TC-pear-NB	2	9/11	4/11	5	1.19

Table 11. Measured OD-values for yeast and mould (YM) samples, cultured first in ready-to-drink pear drink and then transferred to yeast extract peptone dextrose (YPD, Merck). Measurements were taken prior to inoculation into product samples as well as during the development of the medium enrichment. The presented OD-value is the one measured prior to inoculation. The age of the medium differed between the trials and is also presented.

Sample	Trial	Date	Medium renewed	Medium age since renewal [days]	OD -value
YM-pear-YPD	1	1/11	13/10	19	3.92
YM-pear-YPD	2	8/11	4/11	4	3.92

Table 12. Measured OD-values for ACB samples. The samples originates from a previous experiment (data not reported) where an ACB sample cultured in nutrient broth (NB, Merck) was inoculated in ready-to-drink (RTD) pear drink. Measurements were taken prior to inoculation into product samples as well as during the development of the medium enrichment. The presented OD-value is the one measured prior to inoculation. The age of the medium differed between the trials and is also presented.

Sample	Trial	Date	Medium renewed or made	Medium age since renewal [days]	OD -value	Inoculation volume in sample
ACB-NB-RTD	1	31/10	17/10	14	0.74	1 ml
ACB-NB-RTD	2	7/11	31/10	7	0.36	2 ml

5.5 Total Count

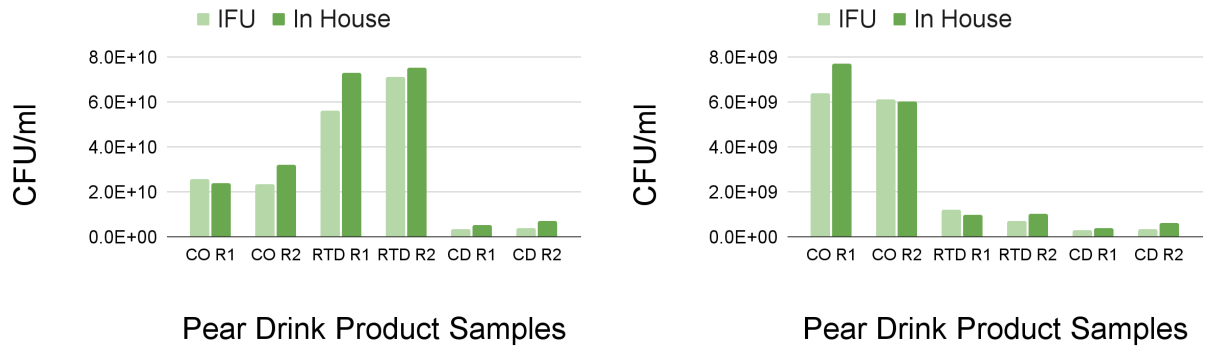
The results from the colony count for Total Count are presented below and tables over the number of counted colonies at the agar plates can be found in Appendix: Results Colony Count. TC-IFU is an abbreviation for the IFU method for Total Count whilst TC-IH is an abbreviation for the In House method for Total Count. Zero-samples were made for both methods and trials and were plated on the same day inoculation in the products occurred. Tables over the zero-samples can be viewed in Appendix: Results Colony Count.

As the growth on previous TC-IH plates (data not shown) had been too numerous to count (TNTC), higher dilutions were plated for Trial 1 for TC-IH (10^{-6}-10^{-8}) compared to TC-IFU (10^{-4}-10^{-6}). For both IFU and IH, the RTD-samples had the highest amount of CFU/ml of approximately 7.0×10^{9}.

For Trial 2, the same dilutions (10^{-4}-10^{-6}) were plated for TC-IFU and TC-IH. For both IFU and IH, the CO-samples had the highest amount of CFU/ml of approximately 6.0×10^{9}.

In Figure 6a (Trial 1) and Figure 6b (Trial 2) below a comparison of CFU/ml between CO, RTD and CD for both IFU and IH is presented in the bar charts. The results chosen to be represented in the figure were those plates which had a colony count between 30 and 300 CFU. However, the

CD TC-IH samples as well as Replicate 1 of the CO TC-IH sample displayed CFU < 30 for Trial 1 due to the too high dilution, but is shown in Figure 6a alongside the other samples for comparison. As seen in Figure 6a and 6b, the concentration CFU/ml observed for CD samples were similar between both the methods and the trials.



***** Trial 1: values for CD R1 and R2 and CO R1 for In House < 30 CFU.

Figure 6a (Total Count Trial 1) and 6b (Total Count Trial 2). A visualisation of the concentration in CFU/ml observed for Total Count, detected with the IFU method and the In House (IH) method. The lighter green bars are the results from IFU whilst the darker green represents the In House-results. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 of the sample and R2 refers to Replicate 2. The IFU-plates (OSA agar) were incubated for 3 days in 30 °C and the IH-plates (PCA agar) were incubated for 5 days in 25 °C.

The plates for Trial 1 displayed large, creamy, white colonies on the RTD agar plates for both methods, whilst CO and CD only had smaller colonies. This can be observed in Figures 7a-c below.

Figure 7a Figure 7b Figure 7c

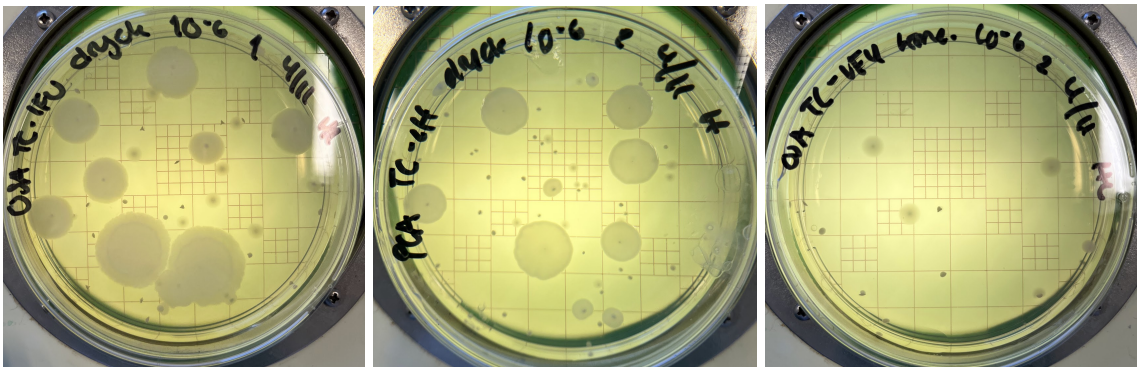
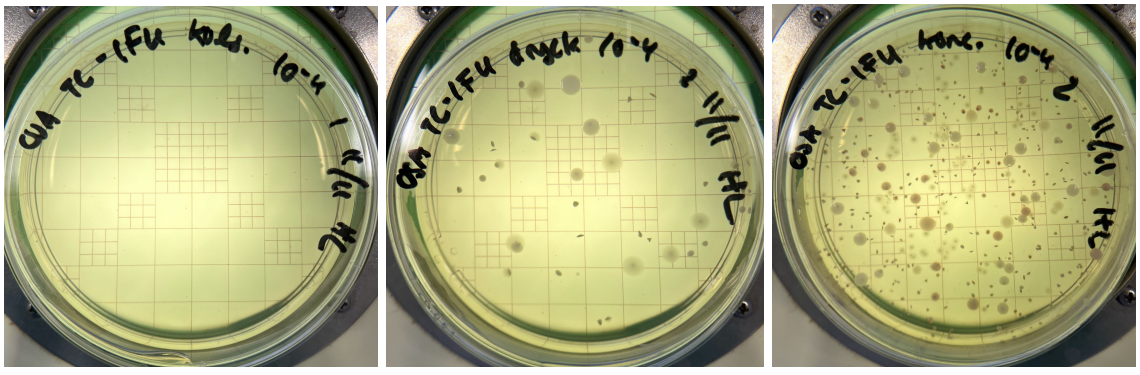


Figure 7a and 7b show agar plates (dilution 10^{-6}) for RTD for TC-IFU and TC-IH Trial 1 where big, creamy, white colonies can be observed. Figure 7c is a reference photo of an CO agar plate (dilution 10^{-6}) for TC-IFU. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. TC-IFU refers to the Total Count detection method provided by IFU and TC-IH refers to the Total Count detection method provided by the Company. The IFU-plates (OSA agar) were incubated for 3 days in 30 ℃ and the IH-plates (PCA agar) were incubated for 5 days in 25 ℃.

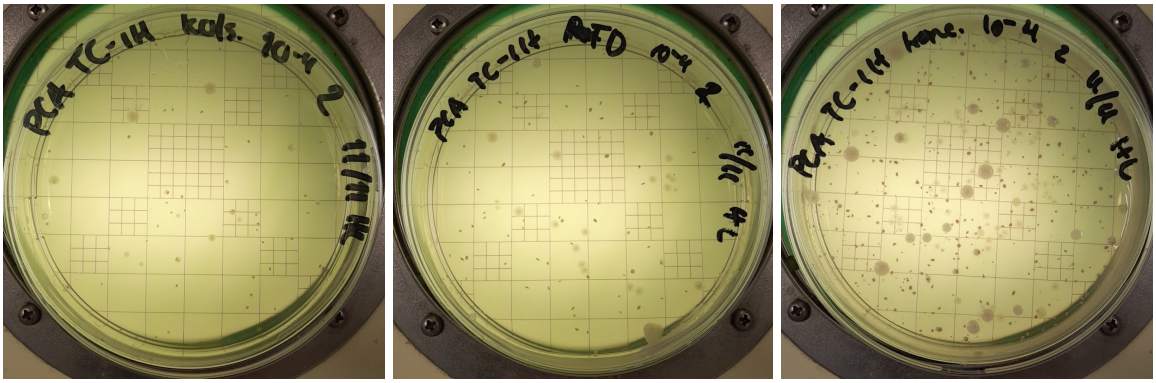
The plates for Trial 2 displayed creamy colonies in various sizes. The CD plates had in general the least amount of colonies and they were also slightly smaller compared to RTD and CO. This can be observed in Figures 8a-c (IFU) and Figures 9a-c (In House) below.

Figure 8a Figure 8b Figure 8c



Figures 8a-c represent the 10^{-4} dilution for CD, RTD and CO respectively for TC-IFU Trial 2. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. TC-IFU refers to the Total Count detection method provided by IFU and plates (OSA agar) were incubated for 3 days in 30 ℃.

Figure 9a Figure 9b Figure 9c



Figures 9a-c represent the 10^{-4} dilution for CD, RTD and CO respectively for TC-IH Trial 2. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. TC-IH refers to the Total Count detection method provided by the Company and the plates (PCA agar) were incubated for 5 days in 25 ℃.

The zero-samples for Trial 1 were made for dilutions 10^{-2}-10^{-4} for IFU which is a lower dilution compared to their corresponding samples (dilutions 10^{-4}-10^{-6}). In Table 13, a comparison between the zero-samples for IFU and their corresponding samples can be observed.

Table 13. A comparison of CFU/ml for the IFU-method for Total Count (TC) between the zero-samples (plated the same day as inoculation in products occurred) and their corresponding samples (plated after two days of incubation in RT). OSA was the medium used. CO stands for diluted pear concentrate, RTD stands for Ready-To-Drink Pear Drink and CD stands for Carbonated Ready-To-Drink Pear Drink. R1 stands for Replicate 1 and R2 stands for Replicate 2. Replicate 1 for carbonated ready-to-drink zero-sample is not included in the table as the plate had too many colonies to count.

Plate	CFU/ml (zero - sample)	CFU/ml (Trial 1)
OSA TC-IFU CO R1	2.7E+07	2.6E+10
OSA TC-IFU CO R2	3.2E+07	2.4E+10
OSA TC-IFU RTD R1	2.4E+07	5.6E+10
OSA TC-IFU RTD R2	2.8E+07	7.1E+10
OSA TC-IFU CDR1	-	3.2E+09
OSA TC-IFU CD R2	2.3E+07	3.9E+09

For IH, dilutions 10^{-8}-10^{-10} were plated, which is a higher dilution compared to their corresponding samples (dilutions 10^{-6}-10^{-8}). All dilutions for the zero-samples gave few or no colonies.

It should be noted that the zero-samples were counted after 2 days, compared to the 3 and 5 days respectively for the IFU-samples and the IH-samples. This was due to both scheduling difficulties (IFU) as they would have been needed to be counted on a Saturday and logistic difficulties (IH) as there was no more room in the heating chambers for the samples.

The zero-samples for Trial 2 were made for dilutions 10^{-2}-10^{-3} for IFU, which is a lower dilution compared to their corresponding samples (dilutions 10^{-4}-10^{-6}). The number of colonies counted on the zero-samples were few or none at all, and the zero-samples were counted after 2 days instead of 3 days due to scheduling difficulties. For IH, dilutions 10^{-2}-10^{-3} were plated, which is a lower dilution compared to their corresponding samples (dilutions 10^{-4}-10^{-6}). The number of colonies counted on the zero-samples were all too numerous to count.

An overview of the results from the detection of Total Count can be observed in Figure 10 where the calculated concentration in CFU/ml for agar plates with a CFU between 30 - 300 is presented.

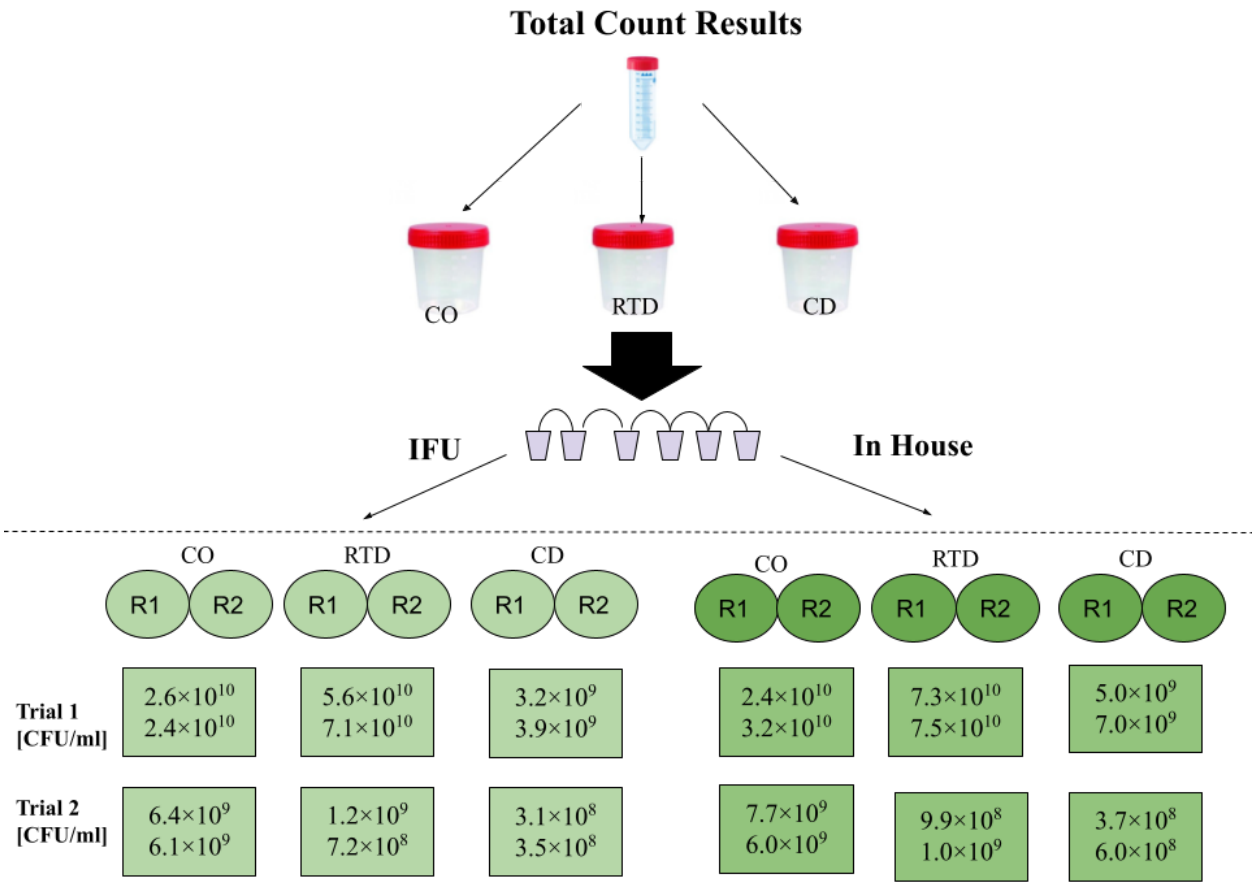


Figure 10. An overview of the results from Total Count where the calculated CFU/ml for Trial 1 and Trial 2 are presented. The results chosen to be represented in the figure were those plates which had a colony count between 30 and 300 CFU. For Trial 1: values for CD R1 and R2 and CO R1 for In House < 30 CFU, but these are still included in the figure for comparison. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 of the sample and R2 refers to Replicate 2. For detailed information regarding the method, see Figure 3 in Methods & Materials. For detailed information regarding the results, see Table 14, 16, 18 and 20 in Appendix: Results Colony Count.

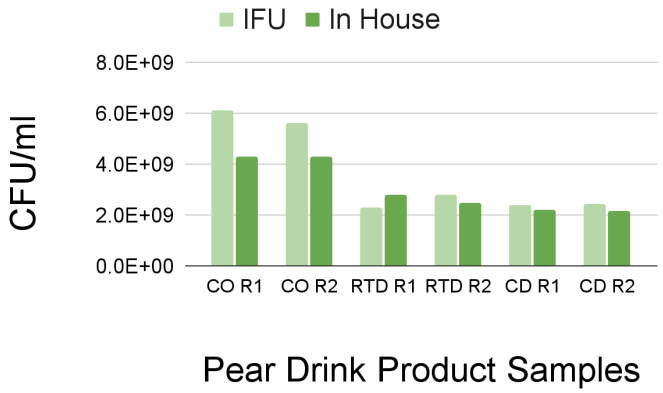
5.6 Yeast & Moulds

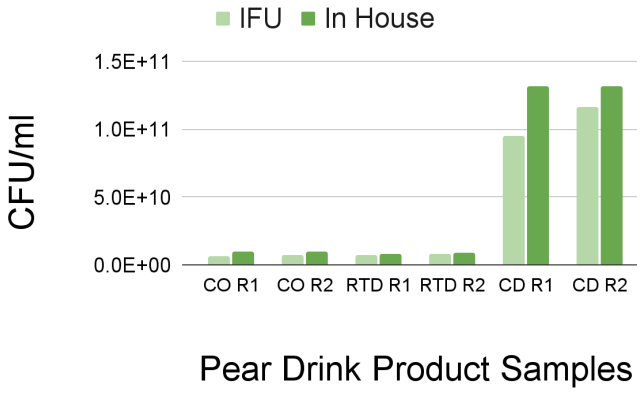
The results from the colony count for Yeast & Moulds are presented below and tables over the number of counted colonies at the agar plates can be found in Appendix: Results Colony Count. YM-IFU is an abbreviation for the IFU method for Yeast & Moulds whilst YM-IH is an abbreviation for the In House method for Yeast & Moulds. Zero-samples were made for both methods and trials and were plated on the same day inoculation in the products occurred. Tables over the zero-samples can be viewed in Appendix: Results Colony Count.

For Trial 1, dilutions 10^{-3}, 10^{-4} and an undiluted sample were plated for YM-IH whilst 10^{-3}-10^{-6} and an undiluted sample were plated for YM-IFU. Both YM-IFU and YM-IH CO-samples had the highest number of CFU/ml. The CO samples for YM-IFU had a concentration of about 6×10^{9} while YM-IH had a concentration of 4.3×10^{9}.

For Trial 2, the same dilutions (10^{-4}-10^{-6}) were plated for YM-IFU and YM-IH. The CD samples had the highest concentration for both methods with approximately 1×10^{11} CFU/ml.

In Figure 11a (Trial 1) and Figure 11b (Trial 2) below a comparison of CFU/ml between CO, RTD and CD for both IFU and IH is presented in the bar chart. The CO YM-IH sample in Trial 1, displayed CFU > 300, and was considered TNTC but is shown in Figure 11a alongside the other samples for comparison. The IFU-samples in Trial 1 were plated with a higher dilution and plates with a colony count between 30 and 300 CFU could therefore be obtained. As seen in Figure 11b, the concentration CFU/ml observed for the CO and RTD samples were similar to each other and between the two methods.



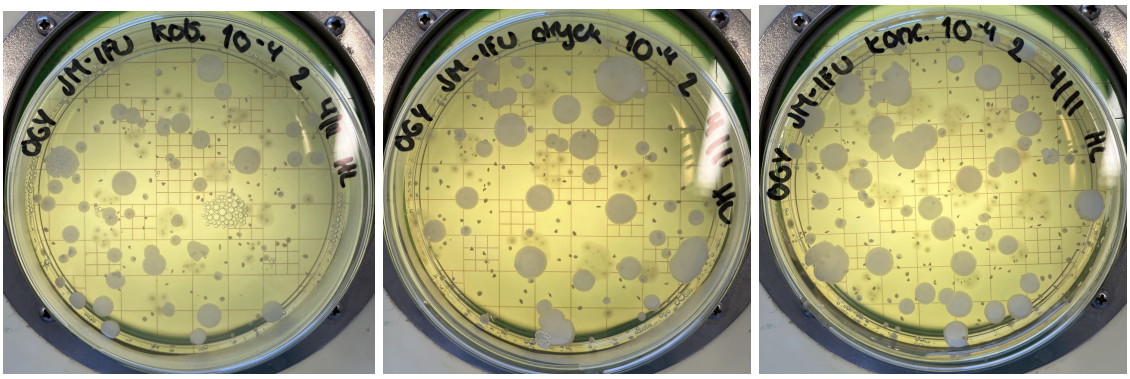


* Trial 1: values for CO R1 and R2 for In House > 300 CFU.

Figure 11a (Yeast & Moulds Trial 1) and 11b (Yeast & Moulds Trial 2). A visualisation of the concentration in CFU/ml observed for Yeast & Moulds, detected with the IFU method and the In House (IH) method. The lighter green bars are the results from IFU whilst the darker green represents the In House-results. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 of the sample and R2 refers to Replicate 2. The IFU-plates (OGY agar) were incubated for 3 days in 30 ℃ and the IH-plates (YGC agar) were incubated for 5 days in 25 ℃.

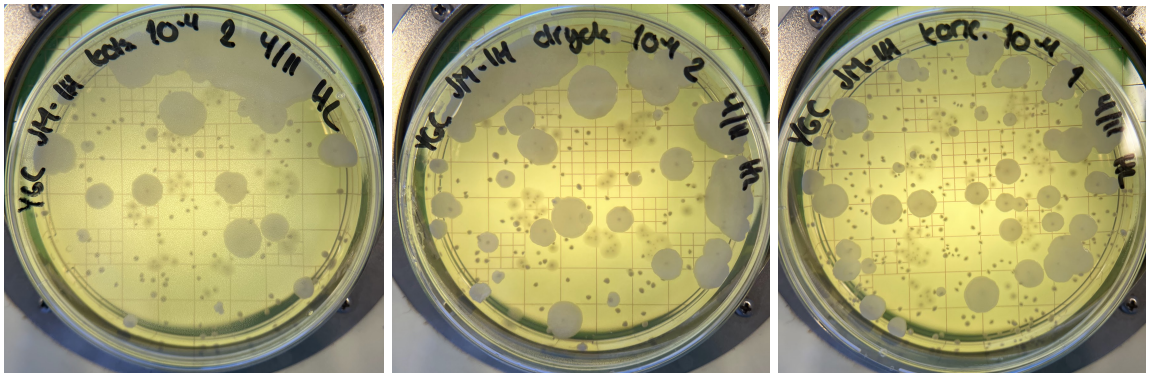
The plates for Trial 1 displayed a mix between large, white colonies and different types of smaller colonies which can be viewed in Figures 12a-c (YM-IFU) and Figures 13a-c (YM-IH).

Figure 12a Figure 12b Figure 12c



Figures 12a-c show dilution 10^{-4} for CD, RTD and CO respectively for YM-IFU Trial 1. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. YM-IFU refers to the Yeast & Moulds detection method provided by IFU and the plates (OGY agar) were incubated for 3 days in 30 ℃.

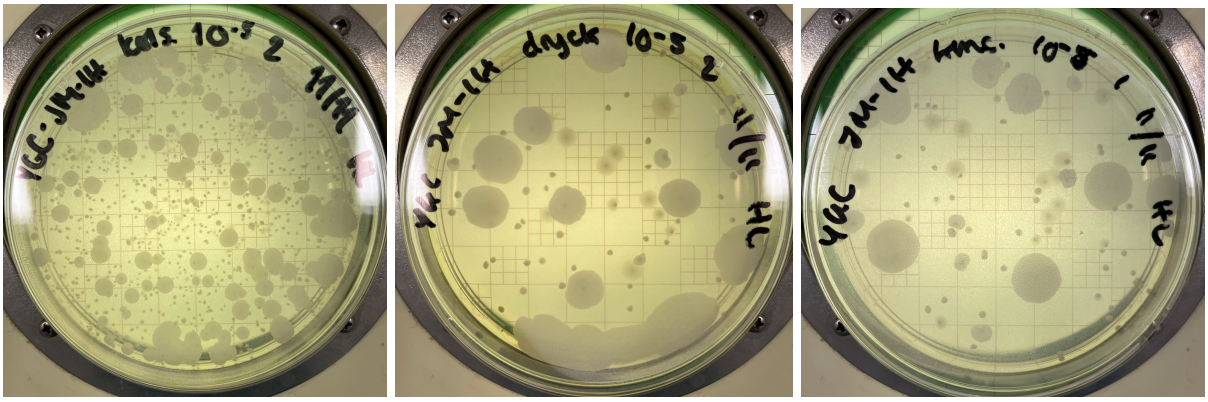
Figure 13a Figure 13b Figure 13c



Figures 13a-c show dilution 10^{-4} for CD, RTD and CO respectively for YM-IH Trial 1. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. YM-IH refers to the Yeast & Moulds detection method provided by the Company and the plates (YGC agar) were incubated for 5 days in 25 °C.

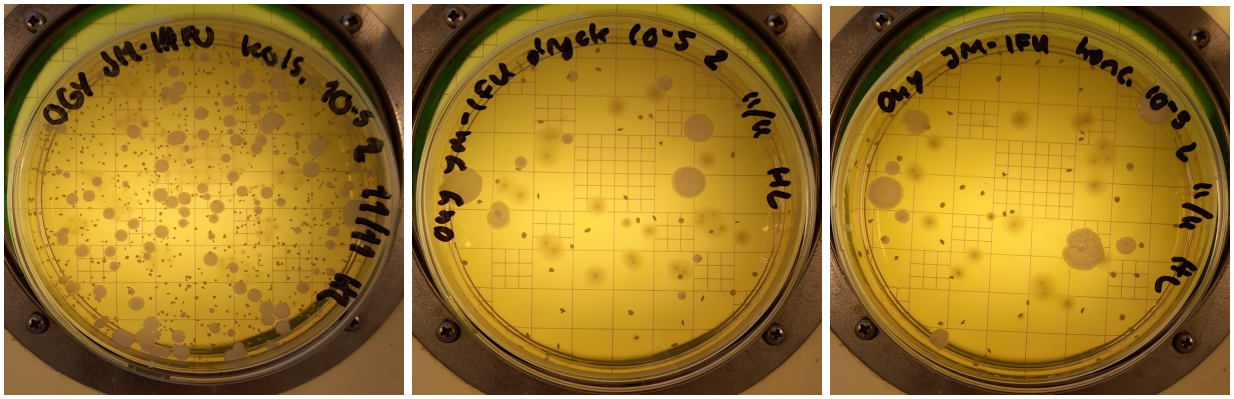
When counting the plates for Trial 2, one single CFU was observed on the 0.9% NaCl control plate that was plated along with YM-IH. However, the 0.9% NaCl control plate for YM-IFU was blank. The IFU and IH plates displayed a mix between large, white colonies and different types of smaller colonies. IH and IFU samples for CD, RTD and CO can be viewed in Figures 14a-c and 15a-c.

Figure 14a Figure 14b Figure 14c



Figures 14a-c show dilution 10^{-5} for CD, RTD and CO respectively for YM-IH Trial 2. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. YM-IH refers to the Yeast & Moulds detection method provided by the Company and the plates (YGC agar) were incubated for 5 days in 25 °C.

Figure 15a Figure 15b Figure 15c



Figures 15a-c show dilution 10^{-5} for CD, RTD and CO respectively for YM-IFU Trial 2. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. YM-IFU refers to the Yeast & Moulds detection method provided by IFU and the plates (OGY agar) were incubated for 3 days in 30 °C.

The zero-samples for both methods for Trial 1 showed no indication of growth. The dilutions 10^{-5}-10^{-8} were plated for YM-IFU while 10^{-4}-10^{-8} were made for YM-IH which are higher than the ones plated for their corresponding samples (dilutions 10^{-3}-10^{-6} and an undiluted sample for YM-IFU and 10^{-3}, 10^{-4} and an undiluted sample for YM-IH). The zero-samples for Trial 2 were made for dilutions 10^{-3} to 10^{-4} for IFU and IH. The concentration of CFU/ml was lower compared to the corresponding samples for CO, RTD and CD. Both CO and RTD had a CFU count below 30. Zero-samples for YM-IH in Trial 2 displayed a lower concentration of CFU/ml for CO and RTD compared to their corresponding samples, while CD had TNTC.

An overview of the results from the detection of Yeast & Moulds can be observed in Figure 16 where the calculated concentration in CFU/ml for agar plates with a CFU between 30 - 300 is presented.

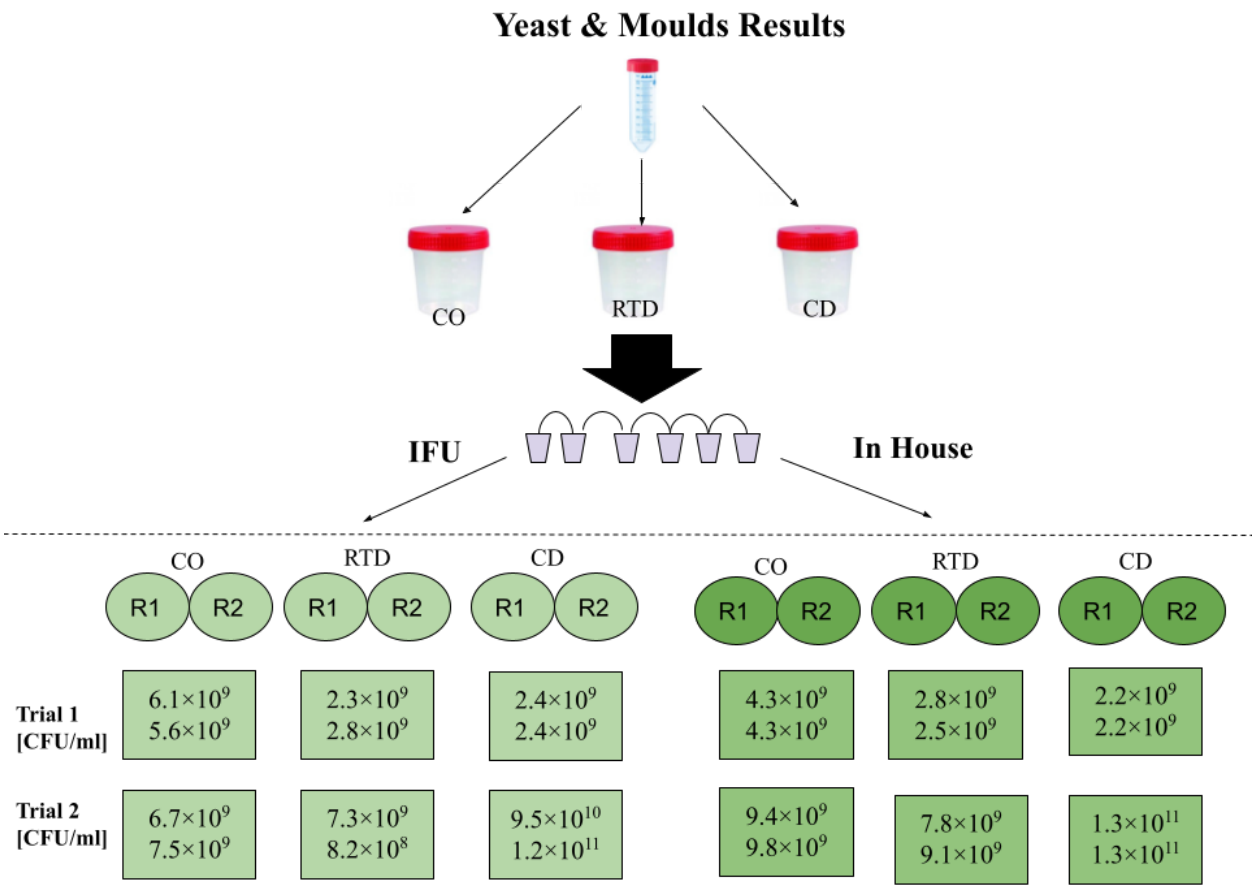


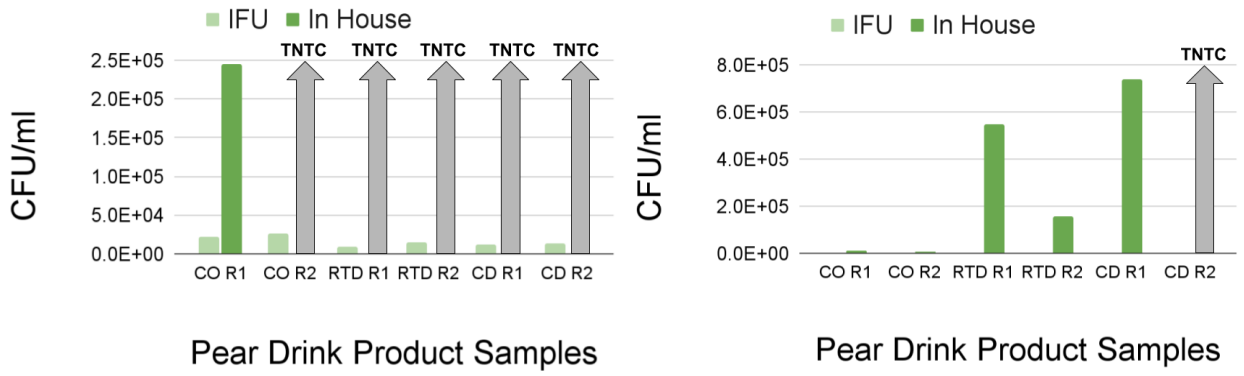
Figure 16. An overview of the results from Yeast & Moulds where the calculated CFU/ml for Trial 1 and Trial 2 are presented. The results chosen to be represented in the figure were those plates which had a colony count between 30 and 300 CFU. For Trial 1: values for CO R1 and R2 for In House > 300 CFU, but these are still included in the figure for comparison. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 of the sample and R2 refers to Replicate 2. For detailed information regarding the method, see Figure 4 in Methods & Materials. For detailed information regarding the results, see Table X and Table X in Appendix: Results Colony Count.

5.7 ACB

The results from the colony count for ACB are presented below and tables over the number of counted colonies at the agar plates can be found in Appendix: Results Colony Count. ACB-IFU is an abbreviation for the IFU method for ACB whilst ACB-IH is an abbreviation for the In House method for ACB. Zero-samples were made for both methods and trials, except for ACB Orange Concentrate, and these were plated on the same day inoculation in the products occurred. Tables over the zero-samples can be viewed in Appendix: Results Colony Count.

For Trial 1 two dilutions (undiluted and 10^{-1}) were plated both for ACB-IFU and ACB-IH. As only one plate for ACB-IH in Trial 1 could be counted as the others were too numerous to count, higher dilutions were plated in Trial 2. The result from both trials are visualised in a bar graph which can be observed in Figure 17a-b. As mentioned, only one plate for ACB-IH in Trial 1 had countable CFU and the other IH-plates which were TNTC are therefore marked in grey. For ACB-IFU in Trial 1, the CO-samples had the highest CFU/ml of approximately 2.5×10^{4}.

In Trial 2, all IFU samples and IH samples are presented in the bar chart in Figure 17b. For ACB-IFU, less than 30 CFU were counted for all samples. ACB-IH had a higher concentration of CFU/ml as seen in Figure 17b. The highest concentration was found for ACB-IH CD. Both replicates displayed a high concentration, but only Replicate 1 had CFU < 300 which could be counted and thus, Replicate 2 for IH is marked with a grey arrow in Figure 17b. The ACB-IFU results are included in the graph in Figure 17b, but because of their low concentration compared to ACB-IH, they are not visible.



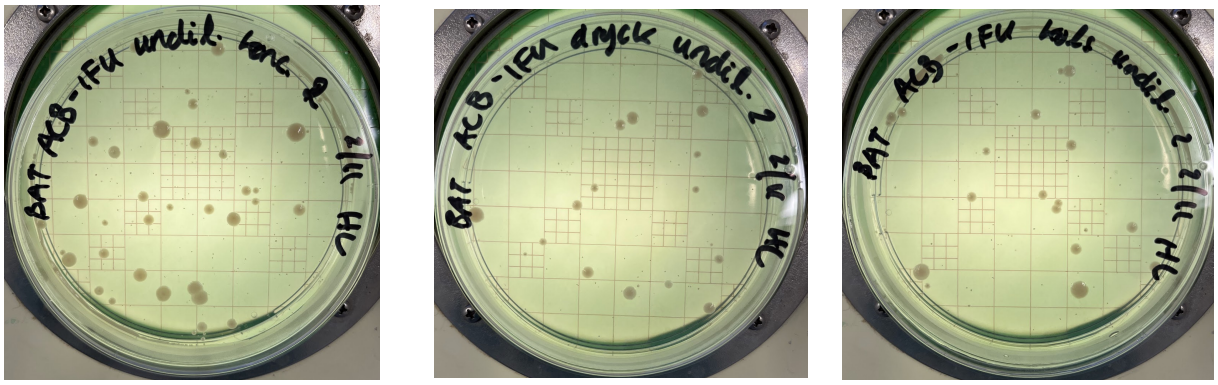
* Trial 1: all samples except CO R1 had CFU > 300 for IH.

* Trial 2: values for CO/RTD/CD R1 and R2 for IFU < 30 CFU. CD R2 for IH > 300 CFU

Figure 17a (ACB Trial 1) and 17b (ACB Trial 2). A visualisation of the concentration in CFU/ml observed for ACB, detected with the IFU method and the In House (IH) method. The lighter green bars are the results from IFU whilst the darker green represents the In House-results. The grey arrows represent the plates which had CFU > 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 of the sample and R2 refers to Replicate 2. The IFU-plates (BAT agar) were incubated for 5 days in 45 ℃ and the IH-plates (OSA agar) were incubated for 2 + 5 days in 45 ℃.

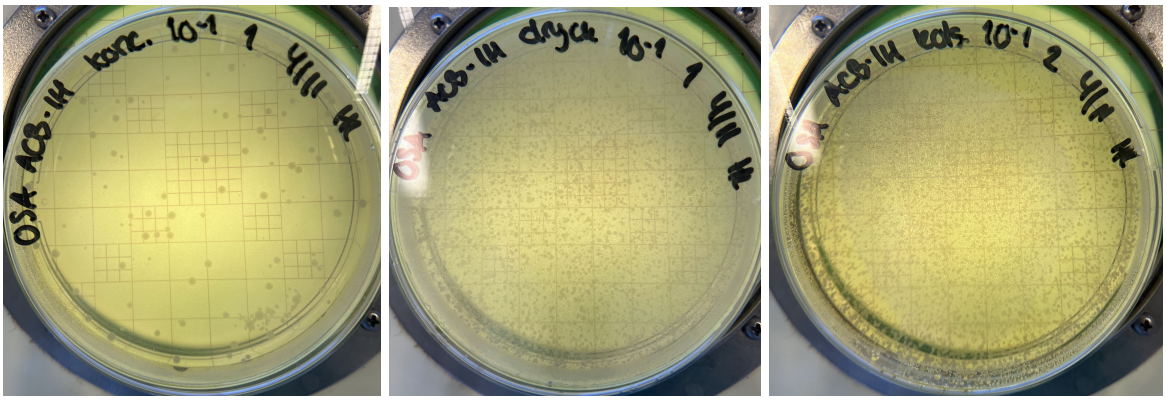
For ACB-IH in Trial 1, it was observed that the CO samples appeared to have fewer colonies than RTD and CD. It was also noted that the plates from ACB-IH had a lot of condensate present on the lid. All plates displayed creamy, beige colonies and these can be observed in Figures 18a-c and Figures 19a-c.

Figure 18a Figure 18b Figure 18c



Figures 18a-c show undiluted samples for CO, RTD and CD respectively for ACB-IFU Trial 1. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. ACB-IFU refers to the ACB detection method provided by IFU and the plates (BAT agar) were incubated for 5 days in 45 ℃.

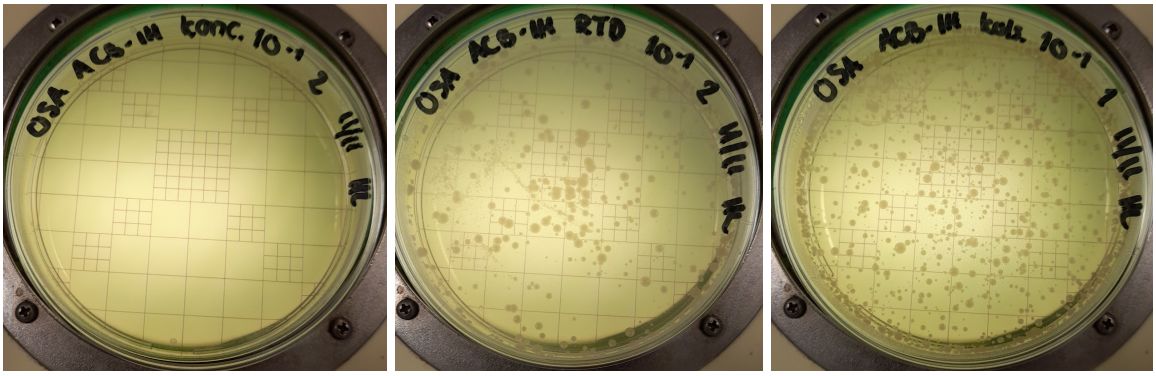
Figure 19a Figure 19b Figure 19c



Figures 19a-c show dilution 10^{-1} for CO, RTD and CD respectively for ACB-IH Trial 1. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. ACB-IH refers to the ACB detection method provided by the Company and the plates (OSA agar) were incubated for 2 + 5 days in 45 ℃.

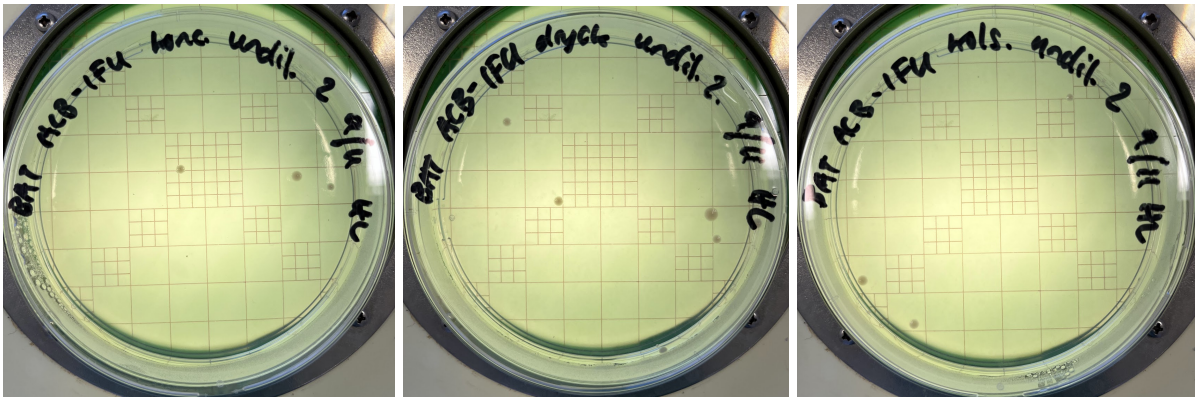
In Trial 2 it was observed that the plates from ACB-IH had a lot of condensate present on the lid. All plates displayed creamy, beige colonies which can be viewed in Figures 20a-c and 21a-c.

Figure 20a Figure 20b Figure 20c



Figures 20a-c show dilution 10^{-1} for CO, RTD and CD respectively for ACB-IH Trial 2. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. ACB-IH refers to the ACB detection method provided by the Company and the plates (OSA agar) were incubated for 2 + 5 days in 45 °C.

Figure 21a Figure 21b Figure 21c



Figures 21a-c show undiluted samples for CO, RTD and CD respectively for ACB-IFU Trial 2. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. ACB-IH refers to the ACB detection method provided by the Company and the plates (OSA agar) were incubated for 2 + 5 days in 45 °C.

For the zero-samples for both IFU and IH in Trial 1, dilutions 10^{-1}, 10^{-2} and undiluted samples were plated. As the 10^{-2} dilutions showed no growth on the plates for the zero-samples this dilution was not used when plating their corresponding samples. For IFU, the zero-samples and their corresponding samples displayed similar results which can be observed in Figure 22. For IH, the zero-samples display growth on the undiluted samples, but no growth at all at higher

dilutions. Their corresponding samples however had too many colonies to count on all plated dilutions.

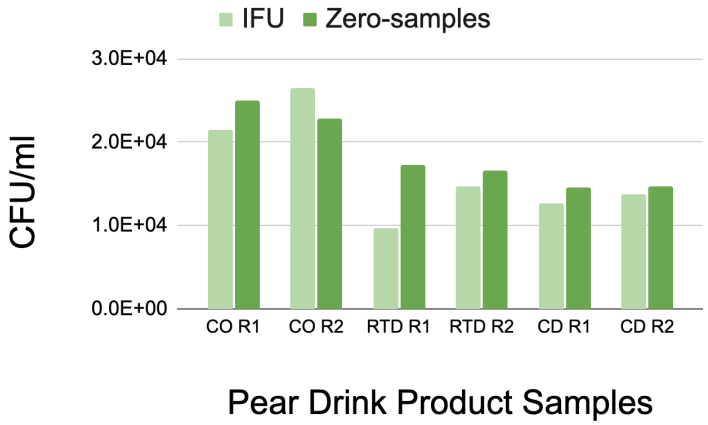


Figure 22 (ACB-IFU Trial 1). A visualisation of the concentration in CFU/ml observed for the zero-samples for ACB and their corresponding samples, detected with the IFU method. The darker green bars represent the zero-samples whilst the light green bars represent their corresponding samples. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 of the sample and R2 refers to Replicate 2. The IFU-plates (BAT agar) were incubated for 5 days in 45 °C.

The zero-samples for ACB-IH and ACB-IFU in Trial 2 were plated for dilution 10^{-1} and undiluted samples. The ACB-IH plates displayed no colonies with the exception of one CD plate which displayed 1 CFU. The zero-samples for ACB-IFU displayed CFU < 30 or no colonies at all. The concentration in the ACB-IFU samples was around 5×10^{2} to 1×10^{3} CFU/ml, which is close to the concentration found for their corresponding samples.

An overview of the results from the detection of ACB can be observed in Figure 23 where the calculated concentration in CFU/ml for agar plates with a CFU between 30 - 300 is presented.

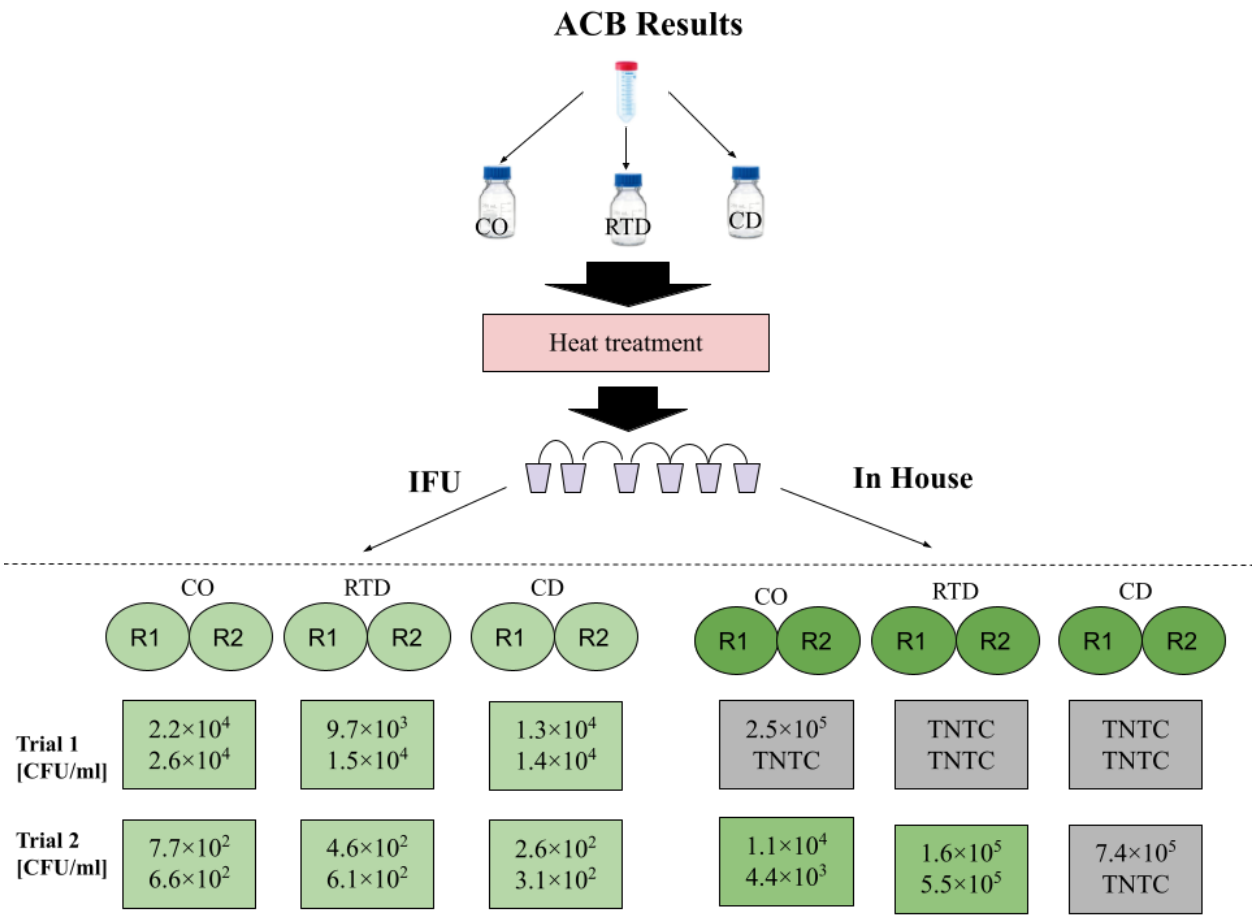
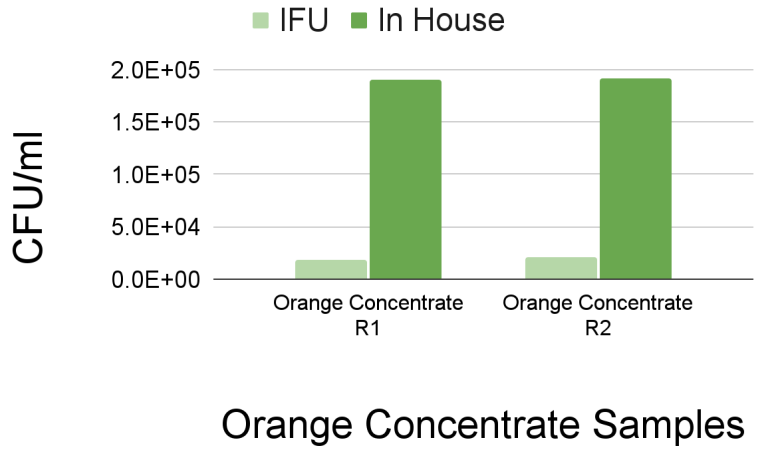


Figure 23. An overview of the results from ACB where the calculated CFU/ml for Trial 1 and Trial 2 are presented. The results chosen to be represented in the figure were those plates which had a colony count between 30 and 300 CFU. For Trial 2: values for CO/RTD/CD R1 and R2 for IFU < 30 CFU. CD R2 for IH > 300 CFU, but these are still included in the figure for comparison. The grey boxes symbolises the plates which had a CFU too numerous to count. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 of the sample and R2 refers to Replicate 2. For detailed information regarding the method, see Figure 5 in Methods & Materials. For detailed information regarding the results, see Table 30, 32, 34 and 36 in Appendix: Results Colony Count.

5.8 ACB Method on Orange Concentrate Sample

The ACB-IFU and ACB-IH methods were also run on a real sample with confirmed ACB contamination from the Company's production. Dilutions 10^{-1}-10^{-3} and an undiluted sample were plated both for ACB-IFU and ACB-IH and the results are visualised in a bar graph which can be observed in Figure 24. For both methods growth only occurred on the undiluted samples, and these are thus the results presented in the graph. It should be noted however that for ACB-IFU

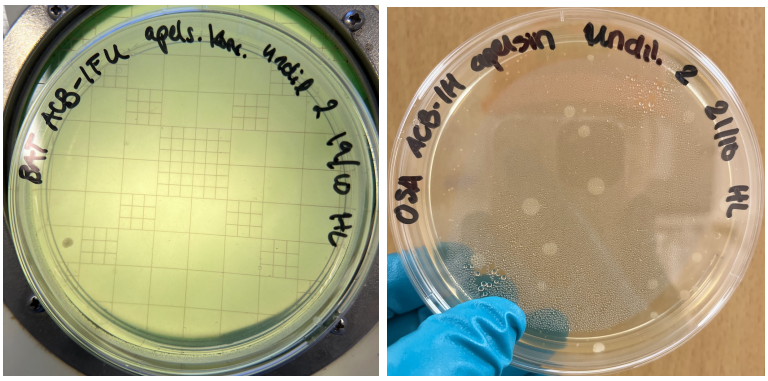
the undiluted samples had less than 30 CFU. Photographs of the plates which displayed creamy, beige colonies can be observed in Figures 25a-b.



* values for R1 and R2 for IFU < 30 CFU.

Figure 24 (ACB Orange Concentrate). A visualisation of the concentration in CFU/ml observed for a real ACB-contaminated orange concentrate sample, detected with the IFU method and the In House (IH) method. The lighter green bars are the results from IFU whilst the darker green represents the In House-results. R1 refers to Replicate 1 of the sample and R2 refers to Replicate 2. The IFU-plates (BAT agar) were incubated for 5 days in 45 °C and the IH-plates (OSA agar) were incubated for 2 + 5 days in 45 °C.

Figure 25a Figure 25b



Figures 25a-b show undiluted orange concentrate samples for ACB-IFU and ACB-IH respectively. ACB-IH refers to the ACB detection method provided by the Company and ACB - IFU refers to the method provided by IFU. The IFU-plates (BAT agar) were incubated for 5 days in 45 °C and the IH-plates (OSA agar) were incubated for 2 + 5 days in 45 °C.

6. Discussion

6.1 Total Count

There is a tendency for similarity in CFU/ml between the IFU and the IH method for the TC samples in both Trial 1 and Trial 2 as seen in Figure 6a and Figure 6b. As can be seen in the graphs, the bars representing IFU and IH are relatively similar in height, which indicates that there is a tendency for these two methods to provide the same results. When observing Figure 10, it is also clear to see that the different methods provide plates with a CFU/ml within the same power of ten for both Trial 1 and 2. This further indicates the tendency for similarity between the two methods. However, since the agar plates for In House CO R1, CD R1 and R2 for Trial 1 had a CFU below 30, these results are deemed less trustworthy than those for Trial 2.

If you compare the bar charts for Trial 1 and 2 (Figure 6a and 6b) there is a clear difference between which inoculated products expressed most growth. In Trial 1, the RTD samples had considerably more growth than both the CD samples and the CO samples. The agar plates for these RTD samples also displayed large, creamy white colonies that were not present on any of the other agar plates which can be seen in Figures 7a-c. These were not present on the samples for Trial 2, and a reason for this might be because the medium used to inoculate the products was renewed between Trial 1 and 2, which can be seen in Table 10. The renewal of the medium could have resulted in a different composition of microorganisms, which would therefore explain the difference in results between Trial 1 and 2. Furthermore, the medium used in Trial 2 was also used only 5 days after its renewal compared to the 20 days for Trial 1, and this could also explain the differences between Trial 1 and 2.

There are several different theories as to why the large white colonies were only present on the RTD samples and not on the CO or CD samples. For instance, the sample could have been contaminated during the experiment as the environment was not completely sterile. Another explanation could be that a higher concentration of microorganisms was pipetted from the inoculated sample which was then used for the serial dilution. The containers in which the samples were present were difficult to mix and it is thereby possible that the concentration could differ if the sample had not been mixed properly. The colonies were not present on the

zero-samples, however these zero-samples were as mentioned counted after only 2 days on incubation and it is thereby possible that the colonies would have appeared on the plates had they been given a longer incubation time.

For Trial 2, the CO samples had considerably more growth than RTD and CD. The colonies on the agar plates for Trial 2 also looked quite similar between CO and RTD, whilst the colonies on the CD samples were much smaller in size which can be seen in Figures 8a-c and 9a-c. The CD samples for both Trial 1 and 2 had the least amount of growth present, and this might be due to the lesser amount of oxygen available for the microorganisms in the product. However, according to Kregiel, aciduric microorganisms such as LAB, AAB, ACB and certain species of yeast and moulds are also capable of spoiling carbonated products. Nevertheless, it is viable to assume after these results that although the microorganisms are capable of growing in carbonated products the growth seems to be more inhibited than for the non-carbonated samples.

It is interesting to compare the zero-samples for IFU for both Trial 1 and 2. Although lower dilutions were used when plating the zero-samples, they showed little or no growth whilst the corresponding samples had considerably more growth, which can be observed in Table 13 for Trial 1. This might indicate that the microorganisms accumulated in the product which they were present in for 2 days before being plated. It should be noted however that the zero-samples for IFU were counted after only 2 days of incubation instead of 3 days which was the case for their corresponding samples. This might therefore also be a cause of the lesser growth on the zero-samples.

The zero-samples for In House Trial 1 had a very high dilution and therefore displayed little or no growth. These samples were however counted after only 2 days instead of 5 since there was no more room in the heating chamber. For Trial 2, lower dilutions were plated for the zero-samples compared to their corresponding samples and all the plates had too many colonies to count after 5 days.

In general, it seems as if the zero-samples for In House had more growth than IFU and a reason for this might be the different agars used. The In House-method uses PCA agar, which is a

non-selective medium, whilst IFU uses OSA agar. OSA is a more selective medium than PCA because of its lower pH (pH 5.4 vs. pH 7.0) and it is therefore more difficult for the microorganisms to grow on this, especially before they have had a chance to recover from being transferred to the products. A viable conclusion is therefore that the difference in results for the zero-samples differ due to the difference of the media used for the methods as well as the incubation time.

6.2 Yeast & Moulds

There is a tendency for similarity in CFU/ml between the IFU and the IH method for the YM samples in both Trial 1 and Trial 2 as seen in Figure 11a-b. When observing Figure 16, it is also clear to see that the different methods provide plates with a CFU/ml within the same power of ten for both Trial 1 and 2. This further indicates the tendency for similarity between the two methods. In Trial 1, the highest concentration of CFU/ml was seen for the CO samples, while RTD and CD were similar to each other. The difference in concentration between the IFU and IH CO-samples could be due to the fact that higher dilutions were plated for IFU compared to IH. The dilutions plated for IH were all considered to be TNTC as the CFU > 300. However, they were still counted and included in Figure 11a to demonstrate that the highest growth observed in the CO-samples was still a trend in Trial 1. If higher dilutions had been plated for IH, it is possible that the concentrations obtained for the CO-samples would be more similar to each other.

In Trial 2, the highest concentration of CFU/ml was found for the CD samples and the CO and RTD were instead comparable to each other. The concentration CFU/ml in the CD samples in Trial 2 was much higher compared to the RTD and CO samples as well as compared to the concentration found for the CD samples in Trial 1. This is true for both the IH and IFU method in both trials respectively. The large difference in concentration between CD and the other products in Trial 2 can be seen in Figures 14a-c and 15a-c. The difference in composition between CD compared to RTD is the difference in pH. Because of the added and dissolved carbon dioxide, the pH for CD was measured to be 3.06 compared to pH 3.13 which was measured for RTD. The pH for the product after inoculation and incubation was never measured, but it could have declined even further for both products depending on the microorganisms

present. The lower pH could thereby aid the growth of certain yeast and moulds. Furthermore, as Kregiel states that yeasts are very tolerant towards carbonation, this could also explain the high CFU values of the CD samples.

The differences observed between Trial 1 and Trial 2 could be because while the origin of the mixed medium culture used was the same for both trials, the trials were conducted two different weeks, and thus the proportion of different microorganisms in the medium in all likelihood varied. Different microorganisms will also have different ability to grow in the liquid product and on the solid agar plate. A different proportion of microorganisms could therefore generate different countable CFU on the plates even though the concentration of cells in the mixed culture remained similar. OD-values for the mixed culture were measured prior to inoculation to obtain an inclination of the cell concentration in the medium. However, there was no possible way with the material at hand to determine which these microorganisms were or if they were still viable. To keep the microorganisms in the mixed culture alive, the medium was renewed using some of the old medium as well as nutrient broth. However, the age of the medium since it had last been renewed differed between the trials as seen in Table 11. This could have affected the proportion of microorganism variants. This difference in the composition of microorganisms in the products does however reflect the reality that the Company faces when performing quality tests.

There was a case of contamination in YM Trial 2. 1 CFU was observed on the 0.9% NaCl control plate made for YM-IH. However, the 0.9% NaCl control plate made for YM-IFU had no growth and the plates made using the highest dilutions for the YM samples, i.e., where the highest ratio of NaCl:YM sample could be found in the dilution series, showed no indication of growth at all. This indicates that the contaminated control plate for NaCl was a contamination that only occurred on that specific plate as no tendency for contamination was observed on the other plates. The results for Trial 2 have therefore been considered to be legitimate and included in the study.

The concentration of CFU/ml for the zero-samples for both trials were lower than the ones found for their corresponding samples for all products with the exception of CD IH Trial 2, which had TNTC and thus, a concentration for this sample could not be estimated. From this, it can be

concluded that the microorganisms have accumulated in the samples during the incubation period of 3 days.

6.3 ACB

As can be observed in Table 12, the OD-values for the ACB samples were quite low for both Trial 1 and 2, and the decision to increase the inoculation volume in the products were therefore made in the hope that the microorganisms would still be able to grow and produce viable results. However, despite being transferred to a renewed medium and left to accumulate for seven days, the ACB sample used for Trial 2 was even lower than for Trial 1, and this was also noticeable in the results, especially for IFU. For Trial 1, the IFU method gave viable results for the undiluted samples for all products where the CFU was between 30 - 300.

As can be seen in Figure 17a-b as well as Figure 23, all samples for the IFU method in Trial 1 and Trial 2 had a similar and comparable concentration to each other. In Trial 2, however, none of the plates for IFU had a CFU over 30 which suggests that the growth had diminished compared to Trial 1. An interesting observation made for the In House-results was that the pear concentrate samples displayed considerably less growth on the agar plates compared to ready-to-drink and carbonated samples. This can easily be observed in Figure 23 where (in Trial 2) the CO-samples had 10 - 10^2 less CFU/ml compared to the RTD and CD samples. For Trial 1, it was only one CO-sample that could be counted as the rest of the samples had too many colonies to count.

A theory of the difference in CFU/ml between the concentrate and the other samples may lie in the composition of the concentrate. As it has a higher °Bx (approximately 11.5) compared to RTD and CD (which both have a °Bx of approximately 7.5) it would be a tougher environment for the bacteria to grow in. This could perhaps also be enhanced by the heat treatment the bacteria is subjected to before their pre-incubation of two days as the bacteria would then also be weakened by the treatment. As mentioned, no such difference between the pear concentrate samples and the others can be observed for the IFU-results. This could be due to the fact that the IFU-samples are plated immediately after the heat treatment and no samples then have the chance to recover and germinate as in the IH method. When observing Figure 23, it is also clear

to see that the power of ten has diminished for the CFU/ml for both the IFU and the In House samples in Trial 2 compared to Trial 1, which suggests that less growth has occurred.

It is evident from both Trial 1 and 2 (Figure 17a-b and Figure 23) as well as the Orange Concentrate Sample (Figure 24) that the IH-method resulted in much higher CFU/ml than the IFU-method and there could be several explanations for these results. Firstly, the most evident difference in the two methods are the types of agars used. The In House-method uses the selective OSA agar whilst IFU uses the selective BAT agar, which is specifically used for the detection of ACB (Avantor, 2022). As can be seen in Table 5, the ingredients between BAT and OSA agar are quite different as the BAT agar contains numerous types of salts not found in OSA. Furthermore, the pH for BAT is 4.0 which is considerably lower than the pH for OSA which is 5.4. As the pH growth range for ACB is around 2.5 - 6.0, the bacteria should be able to grow well on both media. However, as it is quite a wide pH-span it is possible that they would have different growth rates within that range as well which also depends on the type of ACB species present. Moreover, the pH optimum for the agar medium on which to detect ACB has been reported to be 4.0 to ensure specificity (Chang & Kang, 2005). This in combination with its ingredients supports the use of BAT as a suitable agar medium for ACB and makes it viable to assume that it might also be tougher for the microorganisms to grow on this medium compared to OSA. Furthermore, although all colonies on the agar plates looked similar with their beige and creamy appearance, no thorough examination of the colonies was made and it is therefore possible that other microorganisms than ACB grew on especially the OSA agar.

Another major difference between the IFU and IH-method was the 2 days pre-incubation after the heat treatment present in the In House-method. The agar plates for IFU were poured straight after the samples had cooled down after the heat treatment whilst the IH-samples were put back into the heating chamber at 45°C for 2 days before plating. This would give the ACB bacteria a chance to recover after the heat treatment, and they might thereafter be able to grow better on the OSA plates. The heat treatment step is included in the methods to activate and select for spores. They might not have had a chance to germinate in the IFU method before being plated, which would also explain the lower concentration detected for IFU compared to IH.

It is also interesting to note the similarities between the zero-samples and their corresponding samples for IFU which can be observed in Figure 22 for Trial 1. As can bee seen in the graph, the zero-samples had a similar concentration CFU/ml compared to their corresponding samples and some zero-samples even had a higher CFU/ml than its corresponding sample. This differs from the trends seen for TC and YM. For IH however, there was a clear difference between the zero-samples and their corresponding samples where the zero-samples had a lower CFU/ml. This indicates, as for TC and YM, that growth of microorganisms has occurred in the inoculated products. If you compare the results from the zero-samples from IFU with IH for both Trial 1 and 2, the trend is for IFU zero-samples to have a higher CFU/ml even though the corresponding samples have a lower CFU/ml compared to the IH-samples. The reason for this is unclear, since the OSA agar theoretically is a less sensitive agar than the BAT agar and therefore should allow growth of microorganisms more easily. Another reason for the large difference in concentration observed between the IH samples and their corresponding samples is that they were also pre-incubated in 45 ℃ for 2 days whilst the zero-samples were only incubated in the products for 2 days in RT just as for TC and YM. However, while ACB has the ability to grow at room temperature, ACB's growth optimum is between 40-55 ℃ (Pornpukdeewattana et. al., 2019). As they were pre-incubated in their optimum growth temperature, it is to be expected that the IH samples showed a considerably larger concentration compared to their respective zero-samples as well as the IFU samples.

6.4 Challenges in the experimental setup

When performing and designing the trials, there were a few obstacles that had to be overcome. Most importantly, the lab environment in which the trials were conducted was not completely sterile. As no sterile chamber of any kind or laminar flow hood was available in the Microbiological Lab at the Company as well as a lack of Bunsen burner or another kind of ambient gas burner, an alcohol burner (SCHOTT, see Figure 26 in Appendix: Material List) was used instead. While this burner produced a flame and the pipetting of samples was performed in close proximity to the flame, the flame was smaller compared to that of a Bunsen burner. Because of this, there was always a risk of contamination of the samples. For more reliable results, another study continuing on this project could be performed at a facility where a sterile environment can be ensured. This will be further discussed in 6. Further Work.

Furthermore, only three heating chambers were available, and as these were in constant use, only three different temperature settings could be applied as well. As mentioned in the Methods section, the In House method for TC and YM respectively, uses 25 ℃, while both the IFU and IH method for ACB uses 45 ℃. The third temperature chosen was therefore 30 ℃. This is because the IFU method for TC uses 30±2 ℃ and because the heating cabinet used for 30 ℃ could not hold a temperature accurately below 30 ℃. However, the IFU method for YM uses 27±2 ℃. When the trials for IFU YM were conducted, 30 ℃ was used but this meant that the exact protocol for the IFU method was not followed. Moreover, OGY agar has been reported to lose its ability to inhibit bacterial growth when incubated above 25 °C, as this temperature causes hydrolysis of oxytetracycline (Beuchat, 1992). However, another study reported that OGY plates showed growth of yeasts and moulds at temperatures above 25 °C despite this statement (Bovill et. al., 2001). Nevertheless, it seems that 30 °C did not affect the OGY agar in this study, as there was a tendency for similarity in CFU/ml between the IFU and the IH method that was observed for both trials and thus this deviation from the IFU method still produced comparable results.

Another challenge was the limited logistics of the lab. As mentioned previously, the 25 ℃ heating chamber was used for the IH TC and YM trials. But this also meant that it was used by the QC personnel for their routine control of TC and YM in the Company´s products. At times, the heating chamber was completely stocked and decisions had to be made regarding which samples to keep and which to throw away even though the total incubation time had not been reached. This decision was only ever made for zero-samples, and occasionally some of the zero-samples were moved for their last days of incubation to the 30 ℃ heating chamber instead.

A challenge related more closely to the experimental design of the method was that it was difficult to ensure an evenly mixed inoculated sample. As mentioned in the method, 120 ml sterile beakers were used to inoculate 100 ml of either RTD, CD or CO with 100 µl of mixed culture mixture. These beakers were difficult to mix without risking contamination of the sample, and it is therefore possible that a higher or lower concentration of microorganisms were pipetted from the sample The uneven mixing was also a problem when measuring the brix value of the

diluted pear concentrate, which therefore varied between the CO samples between trials as seen in Table 8.

6.5 Challenges when changing method

There are many aspects that must be taken into consideration before a decision can be made for switching from one detection method to another. Firstly, the two methods must be thoroughly compared and tested against each other to determine if they provide similar results, which has been the object of this project.

Secondly, there is also an economic aspect that must be taken into consideration. As production cannot continue until the quality analysis on the tested raw material or product has been finalised it is economically beneficial to have a detection method which requires a shorter amount of time before results are obtained. From an economic point of view, it is also important that the detection methods are trustworthy and do not produce false positive results. If the Company receives a positive result from one of their quality tests, the solution is often to pasteurise the product at a higher temperature to kill any eventual microorganisms that might be present. To pasteurise at a higher temperature is obviously more energy demanding and thereby also more expensive. Should the Company therefore receive a lot of false positive results on their quality tests this would mean a great economic loss that could have been avoided.

However, it is also equally important that the method does not produce false negative results as this could lead to contaminated products being brought out on the market. Whilst the microorganisms present in the Company's products are traditionally safe and only affect the sensory aspects of the products, it could cause a lot of harm to the Company's brand if they were to release a lot of contaminated products. This could also therefore affect the economy of the Company in the long term as well as damage their reputation as a company.

As discussed above under ACB, the In House method obtained a higher concentration of CFU/ml in both trials compared to the IFU method. This could mean that if the concentration of ACB in the sample is low, the IFU method would not detect the presence of ACB while that low concentration could be detected in the In House method. However, it could also be considered at

what concentration ACB would become a spoilage problem. No conclusive studies regarding this have been found, and it is therefore suggested that further studies be made. Furthermore, the presence of ACB does not necessarily mean that enough substances, such as guaiacol, which create the off-odours and off-flavours are produced in an amount that is detectable in the products.

As concluded before, the pre-incubation after the heat treatment of the sample used in the IH method could be a factoring reason as to why the In House method displayed a higher concentration compared to IFU. However, while a pre-incubation period is not included in the IFU method, the method states that if desired by the Company, a similar step could be introduced. In the method's appendix, E.1 General, it is written that the sensitivity of the method could be increased by incubating a "packed ready-to-drink product" at 45°C for "a defined time, the time is to be defined by the lab" (IFU, 2019). Thus, if desired by the Company, and in order to possibly increase the sensitivity of the IFU method to make it comparable to the In House, this step of 1 or 2 days could be included in the method. This will be further discussed in 6. Further Work.

Another important aspect to consider when switching methods is the historical data that could be lost when changing to a new method. It is very valuable for a company to have historical data with previous results to refer back to, and if the method changes it might be difficult to compare data from the old method to the new.

A specific factor for this project that should be considered is the agar types used for the different methods. Especially the OGY agar used for IFU's yeast and moulds method was difficult to find premade and therefore had to be mixed and autoclaved at LTH, unlike the other agar types which could be bought premade in bottles that only had to be heated up and melted before use. Furthermore, the IFU method for Yeast and Moulds states that the pour plate method should be used. However, the only OGY agar found available for purchase was made for spread plating. Another downside with the OGY agar used in this study (Oxoid) was that it could only be made in batches of 500 ml which is considerably more than would normally be used for a routine analysis at the Company. As it is not possible to reuse the agar once its bottle has been opened, a

lot of agar would therefore go to waste which is negative from both an economic and sustainable point of view.

Finally, perhaps the greatest reason for switching methods is the advantages of using a standardised method. At present moment, as the Company uses their own In House-method it is impossible for them to compare and contest results with other companies and raw material producers. This could prove negative for the Company in the future, and in this aspect it would therefore be more favourable for them to switch to a standardised method used by other companies. While this could be greatly beneficial, it is still important to consider the economic aspects and the credibility of the IFU method.

7. Further Work

There are several suggestions to further work which could be conducted after this project in order to clarify certain aspects before potentially switching to the IFU method. An overview of the following recommendations can be viewed in Table 14 below.

Firstly, as there were some issues regarding the sterility of the work environment, it is suggested that the trial could be remade with more replicates and in a more sterile environment to increase the credibility of the results. As the largest differences were seen between the ACB-methods, trials comparing IH and IFU should be performed again. As the IFU-method suggests, a pre-incubation period could be introduced in the method. The time period for this is to be decided by the Company, but 1 or 2 days are suggested to be tried as 2 days are used in the IH method and if 1 day is sufficient, then that would increase the time efficiency of the method. It is possible that if the pre-incubation period is included in the IFU method, it would make IFU- and IH-results more comparable to each other.

Secondly, the challenges with the agar used for the IFU-methods and specifically the OGY agar could be looked into further. A suggestion for the Company would be to reach out to IFU to receive clarification concerning the use of OGY and spread plate vs pour plate. This has been attempted in this project, but no conclusive results have been received from IFU as of yet.

Thirdly, it could be valuable for the Company to perform statistical analysis over their detection of ACB by Veriflow and how often a positive result from Veriflow also gives positive results for a cultivation method. This could provide the Company with information regarding the occurrence of viable ACB in their samples and thereby potentially aid them in their decision in switching methods. From the data currently available from the Company, pie charts over the detection of ACB have been made and can be viewed in Figures 27, 28 and 29 in the Appendix. More data is however necessary in order to obtain trustworthy results.

Finally, it is recommended that the two methods be run parallel for a time in the routine analysis so their comparability could be investigated for a longer period of time with real samples. If

desirable results are obtained and the decision then is made to switch to the IFU method, a suggestion would also be to switch one method at the time to facilitate the switch.

Table 14. The following table summarises the recommendations for the Company's further work and can be used as an aid if the decision to switch from their In House method to IFU's method is made. The changes in agars used, temperature and incubation times are included as well as other notes and recommendations.

Microorganism group	Agar Changes	Temperature Changes	Incubation Time Changes	Execution when changing method
Total Count (IFU)	OSA	**Incubation temperature:** 30±2 ℃	3 days	In parallel with In House method for a period of time
Yeast & Moulds (IFU)	OGY Find a supplier that enables pour plate. Reach out to IFU to inquire about their choice of OGY and clarification regarding pour vs. spread plate.	**Incubation temperature:** 27±2 ℃	3 days, if no growth check again after 5 days	In parallel with In House method for a period of time
ACB (IFU)	BAT	**Incubation temperature:** 45 ℃ (no change in temperature) **Heat treatment:** 80℃ for 10 min	5 days (no change in incubation time)	Further experiments recommended Introduce an incubation period prior to heat treatment according to IFU Microbiological Method no. 12 (Appendix E). We recommend 1-2 days in 45 ℃ (keep product in their packaging during incubation period).

8. Conclusion

To conclude this project, the results obtained show a tendency for similarity between the IFU and IH method for Total Count and Yeast and Moulds. A switch in methods could therefore be made for both of these methods. However, it is suggested to switch one method at a time and a recommendation is to start with Total Count as the agar used in this method (OSA) is already used by the Company and is more accessible than the OGY agar used for Yeast and Moulds.

As the results for the ACB-methods differed substantially more trials should be made before the decision of a switch can be made. See Further Work for suggestions regarding this.

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Appendix

Results Colony Count

Tables from the colony counts can be viewed below. The plates are named based on the following principle: agar name, microorganism - method, product_dilution, Replicate 1 or 2. TNTC - too numerous to count, TFTC - too few to count and blank - no colonies. The Total Dilution Factor is calculated according to Equation 2 and CFU/ml is calculated according to Equation 1.

Pre-trial

Table 15. Results from pre-trial where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. The media used was YGC and the method used was the Company's method for the detection of Yeast & Moulds. TFTC is an abbreviation for Too Few To Count and were plates with CFU < 30. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU > 300.

Plate	Dilution	No. of colonies	Total Dilution Factor	CFU/ml
YGC YM-IH RTD 1 ml R1	10^{-3}	TNTC	10^{5}	-
YGC YM-IH RTD 1 ml R2	10^{-3}	TNTC	10^{5}	-
YGC YM-IH RTD 1 ml R1	10^{-4}	TNTC	10^{6}	-
YGC YM-IH RTD 1 ml R2	10^{-4}	TNTC	10^{6}	-
YGC YM-IH RTD 1 ml R1	10^{-5}	99	10^{7}	9.9×10^{8}
YGC YM-IH RTD 1 ml R2	10^{-5}	114	10^{7}	1.1×10^{9}
YGC YM-IH RTD 1 ml R1	10^{-6}	37	10^{8}	3.7×10^{9}
YGC YM-IH RTD	10^{-6}	TFTC	10^{8}	-

1 ml R2				
YGC YM-IH RTD 1 ml R1	10^{-7}	TFTC	10^{9}	-
YGC YM-IH RTD 1 ml R2	10^{-7}	TFTC	10^{9}	-
YGC YM-IH RTD 0.1 ml R1	10^{-3}	TNTC	10^{6}	-
YGC YM-IH RTD 0.1 ml R2	10^{-3}	TNTC	10^{6}	-
YGC YM-IH RTD 0.1 ml R1	10^{-4}	305/ TNTC	10^{7}	3.1×10^{9}
YGC YM-IH RTD 0.1 ml R2	10^{-4}	265	10^{7}	2.7×10^{9}
YGC YM-IH RTD 0.1 ml R1	10^{-5}	41	10^{8}	4.1×10^{9}
YGC YM-IH RTD 0.1 ml R2	10^{-5}	38	10^{8}	3.8×10^{9}
YGC YM-IH RTD 0.1 ml R1	10^{-6}	TFTC	10^{9}	-
YGC YM-IH RTD 0.1 ml R2	10^{-6}	TFTC	10^{9}	-
YGC YM-IH RTD 0.1 ml R1	10^{-7}	TFTC	10^{10}	-
YGC YM-IH RTD 0.1 ml R2	10^{-7}	TFTC	10^{10}	-

Total Count Trial 1

IFU-method Zero-samples

Table 16. Results from Total Count Zero-samples Trial 1 for the IFU-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 2 days in 30 ℃ and the media used was OSA.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
OSA TC-IFU CO_2 R1	10^2	265	1.00E+05	2.7E+07
OSA TC-IFU CO_2 R2	10^2	323	1.00E+05	3.2E+07
OSA TC-IFU CO_3 R1	10^3	11	1.00E+06	1.1E+07
OSA TC-IFU CO_3 R2	10^3	TNTC	-	-
OSA TC-IFU CO_4 R1	10^4	1	1.00E+07	1.0E+07
OSA TC-IFU CO_4 R2	10^4	0	-	-
OSA TC-IFU RTD_2 R1	10^2	244	1.00E+05	2.4E+07
OSA TC-IFU RTD_2 R2	10^2	278	1.00E+05	2.8E+07
OSA TC-IFU RTD_3 R1	10^3	11	1.00E+06	1.1E+07
OSA TC-IFU RTD_3 R2	10^3	7	1.00E+06	7.0E+06
OSA TC-IFU RTD_4 R1	10^4	0	-	-
OSA TC-IFU RTD_4 R2	10^4	0	-	-
OSA TC-IFU CD_2 R1	10^2	TNTC	-	-
OSA TC-IFU CD_2 R2	10^2	230	1.00E+05	2.3E+07
OSA TC-IFU CD_3 R1	10^3	0	-	-
OSA TC-IFU CD_3 R2	10^3	0	-	-
OSA TC-IFU CD_4 R1	10^4	0	-	-
OSA TC-IFU CD_4 R2	10^4	0	-	-

IFU-method

Table 17. Results from Total Count Trial 1 for the IFU-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 3 days in 30 °C and the media used was OSA.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
OSA TC-IFU CO_4 R1	10^4	TNTC	-	-
OSA TC-IFU CO_4 R2	10^4	TNTC	-	-
OSA TC-IFU CO_5 R1	10^5	258	1.00E+08	2.6E+10
OSA TC-IFU CO_5 R2	10^5	236	1.00E+08	2.4E+10
OSA TC-IFU CO_6 R1	10^6	29	1.00E+09	2.9E+10
OSA TC-IFU CO_6 R2	10^6	18	1.00E+09	1.8E+10
OSA TC-IFU RTD_4 R1	10^4	TNTC	-	-
OSA TC-IFU RTD_4 R2	10^4	TNTC	-	-
OSA TC-IFU RTD_5 R1	10^5	TNTC	-	-
OSA TC-IFU RTD_5 R2	10^5	TNTC	-	-
OSA TC-IFU RTD_6 R1	10^6	56	1.00E+09	5.6E+10
OSA TC-IFU RTD_6 R2	10^6	71	1.00E+09	7.1E+10
OSA TC-IFU CD_4 R1	10^4	310	1.00E+07	3.1E+09
OSA TC-IFU CD_4 R2	10^4	384	1.00E+07	3.8E+09
OSA TC-IFU CD_5 R1	10^5	32	1.00E+08	3.2E+09
OSA TC-IFU CD_5 R2	10^5	39	1.00E+08	3.9E+09
OSA TC-IFU CD_6 R1	10^6	4	1.00E+09	4.0E+09
OSA TC-IFU CD_6 R2	10^6	2	1.00E+09	2.0E+09

IH-method Zero-samples

Table 18. Results from Total Count Zero-samples Trial 1 for the IH-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 2 days in 25 °C and the media used was PCA.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
PCA TC-IH CO_8 R1	10^8	0	-	-
PCA TC-IH CO_8 R2	10^8	0	-	-
PCA TC-IH CO_9 R1	10^9	0	-	-
PCA TC-IH CO_9 R2	10^9	1	-	-
PCA TC-IH CO_10 R1	10^{10}	0	-	-
PCA TC-IH CO_10 R2	10^{10}	0	-	-
PCA TC-IH RTD_8 R1	10^8	0	-	-
PCA TC-IH RTD_8 R2	10^8	0	-	-
PCA TC-IH RTD_9 R1	10^9	0	-	-
PCA TC-IH RTD_9 R2	10^9	0	-	-
PCA TC-IH RTD_10 R1	10^{10}	0	-	-
PCA TC-IH RTD_10 R2	10^{10}	0	-	-
PCA TC-IH CD_8 R1	10^8	0	-	-
PCA TC-IH CD_8 R2	10^8	0	-	-
PCA TC-IH CD_9 R1	10^9	0	-	-
PCA TC-IH CD_9 R2	10^9	0	-	-
PCA TC-IH CD_10 R1	10^{10}	0	-	-
PCA TC-IH CD_10 R2	10^{10}	0	-	-

IH-method

Table 19. Results from Total Count Trial 1 for the IH-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 5 days in 25 °C and the media used was PCA.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
PCA TC-IH CO_6 R1	10^6	24	1.00E+09	2.4E+10
PCA TC-IH CO_6 R2	10^6	32	1.00E+09	3.2E+10
PCA TC-IH CO_7 R1	10^7	2	1.00E+10	2.0E+10
PCA TC-IH CO_7 R2	10^7	1	1.00E+10	1.0E+10
PCA TC-IH CO_8 R1	10^8	0	-	-
PCA TC-IH CO_8 R2	10^8	0	-	-
PCA TC-IH RTD_6 R1	10^6	73	1.00E+09	7.3E+10
PCA TC-IH RTD_6 R2	10^6	75	1.00E+09	7.5E+10
PCA TC-IH RTD_7 R1	10^7	3	1.00E+10	3.0E+10
PCA TC-IH RTD_7 R2	10^7	2	1.00E+10	2.0E+10
PCA TC-IH RTD_8 R1	10^8	1	1.00E+11	1.0E+11
PCA TC-IH RTD_8 R2	10^8	1	1.00E+11	1.0E+11
PCA TC-IH CD_6 R1	10^6	5	1.00E+09	5.0E+09
PCA TC-IH CD_6 R2	10^6	7	1.00E+09	7.0E+09
PCA TC-IH CD_7 R1	10^7	0	-	-
PCA TC-IH CD_7 R2	10^7	1	1.00E+10	1.0E+10
PCA TC-IH CD_8 R1	10^8	0	-	-
PCA TC-IH CD_8 R2	10^8	0	-	-

Total Count Trial 2

IFU-method Zero-samples

Table 20. Results from Total Count Zero-samples Trial 2 for the IFU-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 2 days in 30 ℃ and the media used was OSA.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
OSA TC-IFU CO_2 R1	10^2	7	1.00E+05	7.0E+05
OSA TC-IFU CO_2 R2	10^2	0	-	-
OSA TC-IFU CO_3 R1	10^3	0	-	-
OSA TC-IFU CO_3 R2	10^3	0	-	-
OSA TC-IFU RTD_2 R1	10^2	12	1.00E+05	1.2E+06
OSA TC-IFU RTD_2 R2	10^2	10	1.00E+05	1.0E+06
OSA TC-IFU RTD_3 R1	10^3	5	1.00E+06	5.0E+06
OSA TC-IFU RTD_3 R2	10^3	0	-	-
OSA TC-IFU CD_2 R1	10^2	10	1.00E+05	1.0E+06
OSA TC-IFU CD_2 R2	10^2	12	1.00E+05	1.2E+06
OSA TC-IFU CD_3 R1	10^3	1	1.00E+06	1.0E+06
OSA TC-IFU CD_3 R2	10^3	0	-	-

IFU-method

Table 21. Results from Total Count Trial 2 for the IFU-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 2 days in 30 ℃ and the media used was OSA.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
OSA TC-IFU CO_4 R1	10^4	TNTC	-	-
OSA TC-IFU CO_4 R2	10^4	TNTC	-	-
OSA TC-IFU CO_5 R1	10^5	64	1.00E+08	6.4E+09
OSA TC-IFU CO_5 R2	10^5	61	1.00E+08	6.1E+09
OSA TC-IFU CO_6 R1	10^6	7	1.00E+09	7.0E+09
OSA TC-IFU CO_6 R2	10^6	9	1.00E+09	9.0E+09
OSA TC-IFU RTD_4 R1	10^4	122	1.00E+07	1.2E+09
OSA TC-IFU RTD_4 R2	10^4	72	1.00E+07	7.2E+08
OSA TC-IFU RTD_5 R1	10^5	8	1.00E+08	8.0E+08
OSA TC-IFU RTD_5 R2	10^5	9	1.00E+08	9.0E+08
OSA TC-IFU RTD_6 R1	10^6	0	-	-
OSA TC-IFU RTD_6 R2	10^6	0	-	-
OSA TC-IFU CD_4 R1	10^4	31	1.00E+07	3.1E+08
OSA TC-IFU CD_4 R2	10^4	35	1.00E+07	3.5E+08
OSA TC-IFU CD_5 R1	10^5	5	1.00E+08	5.0E+08
OSA TC-IFU CD_5 R2	10^5	4	1.00E+08	4.0E+08
OSA TC-IFU CD_6 R1	10^6	3	1.00E+09	3.0E+09
OSA TC-IFU CD_6 R2	10^6	0	-	-

IH-method Zero-samples

Table 22. Results from Total Count Zero-samples Trial 2 for the IH-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 2 days in 25 ℃ and the media used was PCA.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
PCA TC-IH CO_2 R1	10^2	TNTC	-	-
PCA TC-IH CO_2 R2	10^2	TNTC	-	-
PCA TC-IH CO_3 R1	10^3	TNTC	-	-
PCA TC-IH CO_3 R2	10^3	TNTC	-	-
PCA TC-IH RTD_2 R1	10^2	TNTC	-	-
PCA TC-IH RTD_2 R2	10^2	TNTC	-	-
PCA TC-IH RTD_3 R1	10^3	TNTC	-	-
PCA TC-IH RTD_3 R2	10^3	TNTC	-	-
PCA TC-IH CD_2 R1	10^2	TNTC	-	-
PCA TC-IH CD_2 R2	10^2	TNTC	-	-
PCA TC-IH CD_3 R1	10^3	TNTC	-	-
PCA TC-IH CD_3 R2	10^3	TNTC	-	-

IH-method

Table 23. Results from Total Count Trial 2 for the IH-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 2 days in 25 ℃ and the media used was PCA.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
PCA TC-IH CO_4 R1	10^4	402	1.00E+07	4.0E+09
PCA TC-IH CO_4 R2	10^4	400	1.00E+07	4.0E+09
PCA TC-IH CO_5 R1	10^5	77	1.00E+08	7.7E+09
PCA TC-IH CO_5 R2	10^5	60	1.00E+08	6.0E+09
PCA TC-IH CO_6 R1	10^6	8	1.00E+09	8.0E+09
PCA TC-IH CO_6 R2	10^6	5	1.00E+09	5.0E+09
PCA TC-IH RTD_4 R1	10^4	99	1.00E+07	9.9E+08
PCA TC-IH RTD_4 R2	10^4	101	1.00E+07	1.0E+09
PCA TC-IH RTD_5 R1	10^5	16	1.00E+08	1.6E+09
PCA TC-IH RTD_5 R2	10^5	6	1.00E+08	6.0E+08
PCA TC-IH RTD_6 R1	10^6	0	-	-
PCA TC-IH RTD_6 R2	10^6	1	1.00E+09	1.0E+09
PCA TC-IH CD_4 R1	10^4	37	1.00E+07	3.7E+08
PCA TC-IH CD_4 R2	10^4	60	1.00E+07	6.0E+08
PCA TC-IH CD_5 R1	10^5	4	1.00E+08	4.0E+08
PCA TC-IH CD_5 R2	10^5	3	1.00E+08	3.0E+08
PCA TC-IH CD_6 R1	10^6	0	-	-
PCA TC-IH CD_6 R2	10^6	0	-	-

Yeast & Moulds Trial 1

IFU-method Zero-samples

Table 24. Results from Yeast & Moulds Zero-samples Trial 1 for the IFU-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 3 days in 30 ℃ and the media used was OGY.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
OGY YM-IFU CO_5 R1	10^5	0	-	-
OGY YM-IFU CO_5 R2	10^5	0	-	-
OGY YM-IFU CO_6 R1	10^6	0	-	-
OGY YM-IFU CO_6 R2	10^6	0	-	-
OGY YM-IFU CO_7 R1	10^7	0	-	-
OGY YM-IFU CO_7 R2	10^7	0	-	-
OGY YM-IFU CO_8 R1	10^8	0	-	-
OGY YM-IFU CO_8 R2	10^8	0	-	-
OGY YM-IFU RTD_5 R1	10^5	0	-	-
OGY YM-IFU RTD_5 R2	10^5	0	-	-
OGY YM-IFU RTD_6 R1	10^6	0	-	-
OGY YM-IFU RTD_6 R2	10^6	0	-	-
OGY YM-IFU RTD_7 R1	10^7	0	-	-
OGY YM-IFU RTD_7 R2	10^7	0	-	-
OGY YM-IFU RTD_8 R1	10^8	1	1.00E+11	1.0E+11
OGY YM-IFU RTD_8 R2	10^8	0	-	-
OGY YM-IFU CD_5 R1	10^5	0	-	-
OGY YM-IFU CD_5 R2	10^5	0	-	-
OGY YM-IFU CD_6 R1	10^6	0	-	-
OGY YM-IFU CD_6 R2	10^6	0	-	-
OGY YM-IFU CD_7 R1	10^7	8	1.00E+10	8.0E+10
OGY YM-IFU CD_7 R2	10^7	0	-	-
OGY YM-IFU CD_8 R1	10^8	0	-	-
OGY YM-IFU CD_8 R2	10^8	0	-	-

IFU-method

Table 25. Results from Yeast & Moulds Trial 1 for the IFU-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 3 days in 30 ℃ and the media used was OGY.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
OGY YM-IFU CO_UNDIL. R1	1	TNTC	-	-
OGY YM-IFU CO_UNDIL. R2	1	TNTC	-	-
OGY YM-IFU CO_3 R1	10^3	TNTC	-	-
OGY YM-IFU CO_3 R2	10^3	TNTC	-	-
OGY YM-IFU CO_4 R1	10^4	397	1.00E+07	4.0E+09
OGY YM-IFU CO_4 R2	10^4	377	1.00E+07	3.8E+09
OGY YM-IFU CO_5 R1	10^5	61	1.00E+08	6.1E+09
OGY YM-IFU CO_5 R2	10^5	56	1.00E+08	5.6E+09
OGY YM-IFU CO_6 R1	10^6	7	1.00E+09	7.0E+09
OGY YM-IFU CO_6 R2	10^6	3	1.00E+09	3.0E+09
OGY YM-IFU RTD_UNDIL. R1	1	TNTC	-	-
OGY YM-IFU RTD_UNDIL. R2	1	TNTC	-	-
OGY YM-IFU RTD_3 R1	10^3	TNTC	-	-
OGY YM-IFU RTD_3 R2	10^3	TNTC	-	-
OGY YM-IFU RTD_4 R1	10^4	230	1.00E+07	2.3E+09
OGY YM- IFU RTD_4 R2	10^4	278	1.00E+07	2.8E+09
OGY YM-IFU RTD_5 R1	10^5	32	1.00E+08	3.2E+09
OGY YM-IFU RTD_5 R2	10^5	22	1.00E+08	2.2E+09
OGY YM-IFU RTD_6 R1	10^6	0	-	-
OGY YM-IFU RTD_6 R2	10^6	3	1.00E+09	3.0E+09
OGY YM-IFU CD_UNDIL. R1	1	TNTC	-	-
OGY YM-IFU CD_UNDIL. R2	1	TNTC	-	-
OGY YM-IFU CD_3 R1	10^3	TNTC	-	-
OGY YM-IFU CD_3 R2	10^3	TNTC	-	-
OGY YM-IFU CD_4 R1	10^4	240	1.00E+07	2.4E+09
OGY YM-IFU CD_4 R2	10^4	244	1.00E+07	2.4E+09

OGY YM-IFU CD_5 R1	10^5	40	1.00E+08	4.0E+09
OGY YM-IFU CD_5 R2	10^5	29	1.00E+08	2.9E+09
OGY YM-IFU CD_6 R1	10^6	2	1.00E+09	2.0E+09
OGY YM-IFU CD_6 R2	10^6	0	-	-

IH-method Zero-samples

Table 26. Results from Yeast & Moulds Zero-samples Trial 1 for the IH-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 6 days in 25 ℃ and the media used was YGC.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
YGC JM-IH CO_4 R1	10^4	1	1.00E+07	1.00E+07
YGC JM-IH CO_4 R2	10^4	0	-	-
YGC JM-IH CO_5 R1	10^5	0	-	-
YGC JM-IH CO_5 R2	10^5	0	-	-
YGC JM-IH CO_6 R1	10^6	0	-	-
YGC JM-IH CO_6 R2	10^6	1	1.00E+09	1.0E+09
YGC JM-IH CO_7 R1	10^7	0	-	-
YGC JM-IH CO_7 R2	10^7	0	-	-
YGC JM-IH CO_8 R1	10^8	0	-	-
YGC JM-IH CO_8 R2	10^8	0	-	-
YGC JM-IH RTD_4 R1	10^4	0	-	-
YGC JM-IH RTD_4 R2	10^4	1	1.00E+07	1.0E+07
YGC JM-IH RTD_5 R1	10^5	1	1.00E+07	1.0E+07
YGC JM-IH RTD_5 R2	10^5	0	-	-
YGC JM-IH RTD_6 R1	10^6	0	-	-
YGC JM-IH RTD_6 R2	10^6	0	-	-
YGC JM-IH RTD_7 R1	10^7	0	-	-
YGC JM-IH RTD_7 R2	10^7	1	1.00E+10	1.0E+10
YGC JM-IH RTD_8 R1	10^8	0	-	-
YGC JM-IH RTD_8 R2	10^8	0	-	-
YGC JM-IH CD_4 R1	10^4	0	-	-
YGC JM-IH CD_4 R2	10^4	0	-	-
YGC JM-IH CD_5 R1	10^5	1	1.00E+08	1.00E+08
YGC JM-IH CD_5 R2	10^5	0	-	-
YGC JM-IH CD_6 R1	10^6	0	-	-
YGC JM-IH CD_6 R2	10^6	0	-	-
YGC JM-IH CD_7 R1	10^7	0	-	-

YGC JM-IH CD_7 R2	10^7	0	-	-
YGC JM-IH CD_8 R1	10^8	0	-	-
YGC JM-IH CD_8 R2	10^8	0	-	-

IH-method

Table 27. Results from Yeast & Moulds Trial 1 for the IH-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 5 days in 25 ℃ and the media used was YGC.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
YGC YM-IH CO_UNDIL. R1	1	TNTC	-	-
YGC YM-IH CO_UNDIL. R2	1	TNTC	-	-
YGC YM-IH CO_3 R1	10^3	TNTC	-	-
YGC YM-IH CO_3 R2	10^3	TNTC	-	-
YGC YM-IH CO_4 R1	10^4	428	1.00E+07	4.3E+09
YGC YM-IH CO_4 R2	10^4	429	1.00E+07	4.3E+09
YGC YM-IH RTD_UNDIL. R1	1	TNTC	1.00E+03	-
YGC YM-IH RTD_UNDIL. R2	1	TNTC	1.00E+03	-
YGC YM-IH RTD_3 R1	10^3	TNTC	1.00E+06	-
YGC YM-IH RTD_3 R2	10^3	TNTC	1.00E+06	-
YGC YM-IH RTD_4 R1	10^4	279	1.00E+07	2.8E+09
YGC YM-IH RTD_4 R2	10^4	249	1.00E+07	2.5E+09
YGC YM-IH CD_UNDIL. R1	1	TNTC	-	-
YGC YM-IH CD_UNDIL. R2	1	TNTC	-	-
YGC YM-IH CD_3 R1	10^3	TNTC	-	-
YGC YM-IH CD_3 R2	10^3	TNTC	-	-
YGC YM-IH CD_4 R1	10^4	218	1.00E+07	2.2E+09
YGC YM-IH CD_4 R2	10^4	217	1.00E+07	2.2E+09

Yeast & Moulds Trial 2

IFU-method Zero-samples

Table 28. Results from Yeast & Moulds Zero-samples Trial 2 for the IFU-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 3 days in 30 ℃ and the media used was OGY.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
OGY YM-IFU CO_3 R1	10^3	17	1.00E+06	1.7E+07
OGY YM-IFU CO_3 R2	10^3	21	1.00E+06	2.1E+07
OGY YM-IFU CO_4 R1	10^4	1	1.00E+07	1.00E+07
OGY YM-IFU CO_4 R2	10^4	1	1.00E+07	1.00E+07
OGY YM-IFU RTD_3 R1	10^3	28	1.00E+06	2.8E+07
OGY YM-IFU RTD_3 R2	10^3	21	1.00E+06	2.1E+07
OGY YM-IFU RTD_4 R1	10^4	31	1.00E+07	3.1E+08
OGY YM-IFU RTD_4 R2	10^4	18	1.00E+07	1.8E+08
OGY YM-IFU CD_3 R1	10^3	TNTC	-	-
OGY YM-IFU CD_3 R2	10^3	288	1.00E+06	2.9E+08
OGY YM-IFU CD_4 R1	10^4	338	1.00E+07	3.4E+09
OGY YM-IFU CD_4 R2	10^4	TNTC	-	-

IFU-method

Table 29. Results from Yeast & Moulds Trial 2 for the IFU-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 3 days in 30 ℃ and the media used was OGY.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
OGY YM-IFU CO_4 R1	10^4	TNTC	-	-
OGY YM-IFU CO_4 R2	10^4	480	1.00E+07	4.8E+09
OGY YM-IFU CO_5 R1	10^5	67	1.00E+08	6.7E+09
OGY YM-IFU CO_5 R2	10^5	75	1.00E+08	7.5E+09
OGY YM-IFU CO_6 R1	10^6	6	1.00E+09	6.0E+09
OGY YM-IFU CO_6 R2	10^6	12	1.00E+09	1.2E+10
OGY YM-IFU RTD_4 R1	10^4	TNTC	-	-
OGY YM-IFU RTD_4 R2	10^4	TNTC	-	-
OGY YM-IFU RTD_5 R1	10^5	73	1.00E+08	7.3E+09
OGY YM-IFU RTD_5 R2	10^5	82	1.00E+08	8.2E+09
OGY YM-IFU RTD_6 R1	10^6	5	1.00E+09	5.0E+09
OGY YM-IFU RTD_6 R2	10^6	8	1.00E+09	8.0E+09
OGY YM-IFU CD_4 R1	10^4	TNTC	-	-
OGY YM-IFU CD_4 R2	10^4	TNTC	-	-
OGY YM-IFU CD_5 R1	10^5	TNTC	-	-
OGY YM-IFU CD_5 R2	10^5	TNTC	-	-
OGY YM-IFU CD_6 R1	10^6	95	1.00E+09	9.5E+10
OGY YM-IFU CD_6 R2	10^6	116	1.00E+09	1.2E+11

IH-method Zero-samples

Table 30. Results from Yeast & Moulds Zero-samples Trial 2 for the IH-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 6 days in 25 ℃ and the media used was YGC.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
YGC YM-IH CO_3 R1	10^3	87	1.00E+06	8.7E+07
YGC YM-IH CO_3 R2	10^3	50	1.00E+06	5.0E+07
YGC YM-IH CO_4 R1	10^4	0	-	-
YGC YM-IH CO_4 R2	10^4	0	-	-
YGC YM-IH RTD_3 R1	10^3	45	1.00E+06	4.5E+07
YGC YM-IH RTD_3 R2	10^3	35	1.00E+06	3.5E+07
YGC YM-IH RTD_4 R1	10^4	34	1.00E+07	3.4E+08
YGC YM-IH RTD_4 R2	10^4	27	1.00E+07	2.7E+08
YGC YM-IH CD_3 R1	10^3	TNTC	-	-
YGC YM-IH CD_3 R2	10^3	TNTC	-	-
YGC YM-IH CD_4 R1	10^4	TNTC	-	-
YGC YM-IH CD_4 R2	10^4	TNTC	-	-

IH-method

Table 31. Results from Yeast & Moulds Trial 2 for the IH-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 5 days in 25 °C and the media used was YGC.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
YGC YM-IH CO_4 R1	10^4	TNTC	-	-
YGC YM-IH CO_4 R2	10^4	TNTC	-	-
YGC YM-IH CO_5 R1	10^5	94	1.00E+08	9.4E+09
YGC YM-IH CO_5 R2	10^5	98	1.00E+08	9.8E+09
YGC YM-IH CO_6 R1	10^6	2	1.00E+09	2.0E+09
YGC YM-IH CO_6 R2	10^6	8	1.00E+09	8.0E+09
YGC YM-IH RTD_4 R1	10^4	TNTC	-	-
YGC YM-IH RTD_4 R2	10^4	TNTC	-	-
YGC YM-IH RTD_5 R1	10^5	78	1.00E+08	7.8E+09
YGC YM-IH RTD_5 R2	10^5	91	1.00E+08	9.1E+09
YGC YM-IH RTD_6 R1	10^6	21	1.00E+09	2.1E+10
YGC YM-IH RTD_6 R2	10^6	6	1.00E+09	6.0E+09
YGC YM-IH CD_4 R1	10^4	TNTC	-	-
YGC YM-IH CD_4 R2	10^4	TNTC	-	-
YGC YM-IH CD_5 R1	10^5	TNTC	-	-
YGC YM-IH CD_5 R2	10^5	TNTC	-	-
YGC YM-IH CD_6 R1	10^6	132	1.00E+09	1.3E+11
YGC YM-IH CD_6 R2	10^6	132	1.00E+09	1.3E+11

ACB Trial 1

IFU-method Zero-samples

Table 32. Results from ACB Zero-samples Trial 1 for the IFU-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 5 days in 45 °C and the media used was BAT.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
BAT ACB-IH CO_UNDIL. R1	1	248	1.01E+02	2.5E+04
BAT ACB-IH CO_UNDIL. R2	1	226	1.01E+02	2.3E+04
BAT ACB-IH CO_1 R1	10^1	44	1.01E+03	4.4E+04
BAT ACB-IH CO_1 R2	10^1	31	1.01E+03	3.1E+04
BAT ACB-IH CO_2 R1	10^2	0	-	-
BAT ACB-IH RTD_ UNDIL. R1	1	171	1.01E+02	1.7E+04
BAT ACB-IH RTD_ UNDIL. R2	1	165	1.01E+02	1.7E+04
BAT ACB-IH RTD_ 1 R1	10^1	49	1.01E+03	4.9E+04
BAT ACB-IH RTD_ 1 R2	10^1	44	1.01E+03	4.4E+04
BAT ACB-IH RTD_ 2 R1	10^2	0	-	-
BAT ACB-IH CD_UNDIL. R1	1	144	1.01E+02	1.5E+04
BAT ACB-IH CD_UNDIL. R2	1	146	1.01E+02	1.5E+04
BAT ACB-IH CD_1 R1	10^1	38	1.01E+03	3.8E+04
BAT ACB-IH CD_1 R2	10^1	41	1.01E+03	4.1E+04
BAT ACB-IH CD_2 R1	10^2	0	-	-

IFU-method

Table 33. Results from ACB Trial 1 for the IFU-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 5 days in 45 °C and the media used was BAT.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
BAT ACB-IFU CO_UNDIL. R1	1	213	1.01E+02	2.2E+04
BAT ACB-IFU CO_UNDIL. R2	1	262	1.01E+02	2.6E+04
BAT ACB-IFU CO_1 R1	10^1	26	1.01E+03	2.6E+04
BAT ACB-IFU CO_1 R2	10^1	41	1.01E+03	4.1E+04
BAT ACB-IFU RTD_UNDIL. R1	1	96	1.01E+02	9.7E+03
BAT ACB-IFU RTD_UNDIL. R2	1	145	1.01E+02	1.5E+04
BAT ACB-IFU RTD_1 R1	10^1	15	1.01E+03	1.5E+04
BAT ACB-IFU RTD_1 R2	10^1	15	1.01E+03	1.5E+04
BAT ACB-IFU CD_UNDIL. R1	1	125	1.01E+02	1.3E+04
BAT ACB-IFU CD_UNDIL. R2	1	136	1.01E+02	1.4E+04
BAT ACB-IFU CD_1 R1	10^1	10	1.01E+03	1.0E+04
BAT ACB-IFU CD_1 R2	10^1	21	1.01E+03	2.1E+04

IH-method Zero-samples

Table 34. Results from ACB Zero-samples Trial 1 for the IH-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 2 + 5 days in 45 ℃ and the media used was OSA.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
OSA ACB-IH CO_UNDIL. R1	1	231	1.01E+02	2.3E+04
OSA ACB-IH CO_UNDIL. R2	1	41	1.01E+02	4.1E+03
OSA ACB-IH CO_1 R1	10^1	0	-	-
OSA ACB-IH CO_1 R2	10^1	0	-	-
OSA ACB-IH CO_2 R1	10^2	0	-	-
OSA ACB-IH RTD_ UNDIL. R1	1	32	1.01E+02	3.2E+03
OSA ACB-IH RTD_ UNDIL. R2	1	0	-	-
OSA ACB-IH RTD_ 1 R1	10^1	0	-	-
OSA ACB-IH RTD_ 2 R2	10^1	0	-	-
OSA ACB-IH RTD_ 2 R1	10^2	0	-	-
OSA ACB-IH CD_UNDIL. R1	1	383	1.01E+02	3.9E+04
OSA ACB-IH CD_UNDIL. R2	1	131	1.01E+02	1.3E+04
OSA ACB-IH CD_1 R1	10^1	0	-	-
OSA ACB-IH CD_1 R2	10^1	0	-	-
OSA ACB-IH CD_2 R1	10^2	0	-	-

IH-method

Table 35. Results from ACB Trial 1 for the IH-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 2 + 5 days in 45 ℃ and the media used was OSA.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
OSA ACB-IH CO_UNDIL. R1	1	TNTC	-	-
OSA ACB-IH CO_UNDIL. R2	1	TNTC	-	-
OSA ACB-IH CO_1 R1	10^1	243	1.01E+03	2.5E+05
OSA ACB-IH CO_1 R2	10^1	TNTC	-	-
OSA ACB-IH RTD_UNDIL. R1	1	TNTC	-	-
OSA ACB-IH RTD_UNDIL. R2	1	TNTC	-	-
OSA ACB-IH RTD_1 R1	10^1	TNTC	-	-
OSA ACB-IH RTD_1 R2	10^1	TNTC	-	-
OSA ACB-IH CD_UNDIL. R1	1	TNTC	-	-
OSA ACB-IH CD_UNDIL. R2	1	TNTC	-	-
OSA ACB-IH CD_1 R1	10^1	TNTC	-	-
OSA ACB-IH CD_1 R2	10^1	TNTC	-	-

ACB Trial 2

IFU-method Zero-samples

Table 36. Results from ACB Zero-samples Trial 2 for the IFU-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 5 days in 45 °C and the media used was BAT.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
BAT ACB-IFU CO_undil R1	1	15	5.10E+01	7.7E+02
BAT ACB-IFU CO_undil R2	1	18	5.10E+01	9.2E+02
BAT ACB-IFU CO_1 R1	10	2	5.10E+02	1.0E+03
BAT ACB-IFU CO_1 R2	10	0	-	-
BAT ACB-IFU RTD_undil R1	1	11	5.10E+01	5.6E+02
BAT ACB-IFU RTD_undil R2	1	16	5.10E+01	8.2E+02
BAT ACB-IFU RTD_1 R1	10	0	-	-
BAT ACB-IFU RTD_1 R2	10	0	-	-
BAT ACB-IFU CD_undil R1	1	23	5.10E+01	1.2E+03
BAT ACB-IFU CD_undil R2	1	11	5.10E+01	5.6E+02
BAT ACB-IFU CD_1 R1	10	0	-	-
BAT ACB-IFU CD_1 R2	10	1	5.10E+02	5.1E+02

IFU-method

Table 37. Results from ACB Trial 2 for the IFU-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 5 days in 45 °C and the media used was BAT.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
BAT ACB-IFU CO_undil R1	1	15	5.10E+01	7.7E+02
BAT ACB-IFU CO_undil R2	1	13	5.10E+01	6.6E+02
BAT ACB-IFU CO_1 R1	10^1	0	-	-
BAT ACB-IFU CO_1 R2	10^1	2	5.10E+02	1.0E+03
BAT ACB-IFU CO_2 R1	10^2	0	-	-
BAT ACB-IFU CO_2 R2	10^2	0	-	-
BAT ACB-IFU RTD_undil R1	1	9	5.10E+01	4.6E+02
BAT ACB-IFU RTD_undil R2	1	12	5.10E+01	6.1E+02
BAT ACB-IFU RTD_1 R1	10^1	0	-	-
BAT ACB-IFU RTD_1 R2	10^1	1	5.10E+02	5.1E+02
BAT ACB-IFU RTD_2 R1	10^2	0	-	-
BAT ACB-IFU RTD_2 R2	10^2	0	-	-
BAT ACB-IFU CD_undil R1	1	5	5.10E+01	2.6E+02
BAT ACB-IFU CD_undil R2	1	6	5.10E+01	3.1E+02
BAT ACB-IFU CD_1 R1	10^1	0	-	-
BAT ACB-IFU CD_1 R2	10^1	0	-	-
BAT ACB-IFU CD_2 R1	10^2	0	-	-
BAT ACB-IFU CD_2 R2	10^2	0	-	-

IH-method Zero-samples

Table 38. Results from ACB Zero-samples Trial 2 for the IH-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 2 + 5 days in 45 °C and the media used was OSA.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
OSA ACB-IH CO_UNDIL. R1	1	0	-	-
OSA ACB-IH CO_UNDIL. R2	1	0	-	-
OSA ACB-IH CO_1 R1	10^1	0	-	-
OSA ACB-IH CO_1 R2	10^1	0	-	-
OSA ACB-IH RTD_UNDIL. R1	1	0	-	-
OSA ACB-IH RTD_UNDIL. R2	1	0	-	-
OSA ACB-IH RTD_1 R1	10^1	0	-	-
OSA ACB-IH RTD_1 R2	10^1	0	-	-
OSA ACB-IH CD_UNDIL. R1	1	0	-	-
OSA ACB-IH CD_UNDIL. R2	1	0	-	-
OSA ACB-IH CD_1 R1	10^1	1	5.10E+02	5.1E+02
OSA ACB-IH CD_1 R2	10^1	0	-	-

IH-method

Table 39. Results from ACB Trial 2 for the IH-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. CO is an abbreviation for Concentrate, RTD is an abbreviation for Ready-To-Drink and CD is an abbreviation for Carbonated Ready-To-Drink. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 2 + 5 days in 45 ℃ and the media used was OSA.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
OSA ACB-IH CO_undil R1	1	211	5.10E+01	1.1E+04
OSA ACB-IH CO_undil R2	1	86	5.10E+01	4.4E+03
OSA ACB-IH CO_1 R1	10^1	53	5.10E+02	2.7E+04
OSA ACB-IH CO_1 R2	10^1	0	-	-
OSA ACB-IH CO_2 R1	10^2	0	-	-
OSA ACB-IH CO_2 R2	10^2	0	-	-
OSA ACB-IH CO_3 R1	10^3	0	-	-
OSA ACB-IH CO_3 R2	10^3	0	-	-
OSA ACB-IH RTD_undil R1	1	TNTC	-	-
OSA ACB-IH RTD_undil R2	1	TNTC	-	-
OSA ACB-IH RTD_1 R1	10^1	TNTC	-	-
OSA ACB-IH RTD_1 R2	10^1	312	5.10E+02	1.6E+05
OSA ACB-IH RTD_2 R1	10^2	107	5.10E+03	5.5E+05
OSA ACB-IH RTD_2 R2	10^2	31	5.10E+03	1.6E+05
OSA ACB-IH RTD_3 R1	10^3	5	5.10E+04	2.6E+05
OSA ACB-IH RTD_3 R2	10^3	0	-	-
OSA ACB-IH CD_undil R1	1	TNTC	-	-
OSA ACB-IH CD_undil R2	1	TNTC	-	-
OSA ACB-IH CD_1 R1	10^1	TNTC	-	-
OSA ACB-IH CD_1 R2	10^1	TNTC	-	-
OSA ACB-IH CD_2 R1	10^2	145	5.10E+03	7.4E+05
OSA ACB-IH CD_2 R2	10^2	TNTC	-	-
OSA ACB-IH CD_3 R1	10^3	0	-	-
OSA ACB-IH CD_3 R2	10^3	0	-	-

ACB Orange Concentrate

IFU-method

Table 40. Results from ACB Orange Concentrate sample for the IFU-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 5 days in 45 °C and the media used was BAT.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
BAT ACB-IFU ORANGE_UNDIL. R1	1	18	1.00E+03	1.8E+04
BAT ACB-IFU ORANGE_UNDIL. R2	1	21	1.00E+03	2.1E+04
BAT ACB-IFU ORANGE_1 R1	10^1	0	-	-
BAT ACB-IFU ORANGE_1 R2	10^1	0	-	-
BAT ACB-IFU ORANGE_2 R1	10^2	0	-	-
BAT ACB-IFU ORANGE_2 R2	10^2	0	-	-
BAT ACB-IFU ORANGE_3 R1	10^3	0	-	-
BAT ACB-IFU ORANGE_3 R2	10^3	0	-	-

IH-method

Table 41. Results from ACB Orange Concentrate sample for the IH-method where the name of the plate, the dilution of the sample, the number of colonies counted on the plate, the calculated Total Dilution Factor and the calculated concentration in CFU/ml is reported. TNTC is an abbreviation for Too Numerous To Count and were plates with CFU >> 300. R1 refers to Replicate 1 and R2 refers to Replicate 2. The plates were incubated for 2 + 5 days in 45 °C and the media used was OSA.

Plate	Dilution Factor	No. of CFU	Total Dilution	CFU/ml
OSA ACB-IH ORANGE_UNDIL. R1	1	190	1.00E+03	1.9E+05
OSA ACB-IH ORANGE_UNDIL. R2	1	191	1.00E+03	1.9E+05
OSA ACB-IH ORANGE_1 R1	10^1	0	-	-
OSA ACB-IH ORANGE_1 R2	10^1	0	-	-
OSA ACB-IH ORANGE_2 R1	10^2	0	-	-
OSA ACB-IH ORANGE_2 R2	10^2	0	-	-
OSA ACB-IH ORANGE_3 R1	10^3	0	-	-
OSA ACB-IH ORANGE_3 R2	10^3	0	-	-

Preparation of mixed stature culture media for TC and YM

In Table 42, the OD values for the samples prepared for making the mixed starter culture media are presented. The samples with the highest OD values for TC and YM respectively were YM-RTD-YPD (YM in inoculated pear drink that was re-inoculated into YPD medium) and TC-RTD-NB (TC in inoculated pear drink that was re-inoculated into nutrient broth).

Table 42. The results from the OD_{620}-measurements made on samples during the pre-trial as well as the numeration of said samples. The Origin column refers to where the sample was collected before it was cultured in a new medium. The OD-value is calculated from a 10^{-1}-dilution and only one measurement per sample was performed. TC is an abbreviation for Total Count, YM is an abbreviation for Yeast & Moulds, NB is an abbreviation for Nutrient Broth and YPD is an abbreviation for Yeast Extract Peptone Dextrose.

Sample Number	Microorganism	Origin	Medium	OD_{620}
1	TC	Plate	NB	0.650
2	TC	Plate	Pear Drink	0.950
3	TC	Inoculated pear drink	NB	2.85
4	TC	Inoculated pear drink	Pear Drink	1.23
5	TC	Inoculated NB	NB	1.90
6	TC	Inoculated NB	Pear Drink	0.940
7	YM	Inoculated NB	NB	0.400
8	YM	Inoculated NB	Pear Drink	0.0300
9	YM	Inoculated NB	YPD	0.660
10	YM	Inoculated pear drink	NB	2.25
11	YM	Inoculated pear drink	Pear Drink	2.20
12	YM	Inoculated pear drink	YPD	5.17
13	Moulds	Sample from the Company's fridge, "Smultron"	NB	1.62
14	Moulds	Sample from the Company's fridge, "Smultron"	Pear Drink	1.09

15	Moulds	Sample from the Company's fridge, "Smultron"	YPD	4.96
16	Yeast	Sample from the Company's fridge, "Årets Skörd"	NB	1.64
17	Yeast	Sample from the Company's fridge, "Årets Skörd"	Pear Drink	1.92
18	Yeast	Sample from the Company's fridge, "Årets Skörd"	YPD	6.50

Material List

Table 43. List of materials used in the project, their manufacturer as well as specifications.

Product	Manufacturer/model	Specifications
Water bath	OneMed Water Bath TECHNE TE-10A Tempette®	8 L T range: -20 - 95 ℃
Autoclave	CERTOCLAV - Tisch-Autoclav CV-EL	12 L / 18 L, LGA Nürnberg, 1900 W/230 V ~ 50 Hz
Scale	Sartorius	max weight 4240 g
Vortex	Scientific Industries	
Heating cabinet 25 ℃	Memmert G1-51	
Heating cabinet 45 ℃	LabRum Klimat	
Heating cabinet 30 ℃	Pol-Eko Apparatus	
10 ml sterile pipettes	Sarstedt	
Peleus ball	VWR chemicals	
Pot for heating agar	Æternum	
Heating Plate	EuroKera	
Cuvettes	BRAND GMBH + CO KG	2.5 ml
Spectrophotometer DR 2800	Hach Lange	
Brix refractometer	Bellingham + Stanley™ RFM340+ Refractometer	
Petri dish	Steris	9 cm
Glass bottle	LABsolute	250 ml
Glass bottle	Simax Czech Repub.	500 ml

Glass bottle	VWR chemicals	1000 ml
Sterile plastic beaker	LABsolute	120 ml
Sterile falcon tube	VWR chemicals	100 ml
Sterile plastic streakers	VWR chemicals	
Sterile pipette tips	Sartorius	1 ml
Sterile straw tips	VWR chemicals	1 ml
Automatic pipette	Sartorius	50 µl - 1000 µl
Pipette	Hirschmann	100 µl - 1000 µl
Dilution pipette	VWR chemicals	1 ml
Colony counter	Dr. N. Gerber Original	
Counting pen	VWR	
Lighter	LABsolute	
Alcohol burner	SCHOTT	
Autoclave plastic bags	VWR chemicals	
Thermometer	ALLA France	

Photograph of the alcohol burner used.

Figure 26. Alcohol burner (SCHOTT)

Agar Medium Compositions

Table 44. Extensive ingredient list with their functionalities for BAT and OSA agar, used for the detection of ACB. The OSA agar is used for the In House-method whilst the BAT agar is used for the IFU-method. (IFU, 2019) (Scharlau Microbiology, 2022)

Ingredients	BAT	OSA	Functionality
Yeast extract	2.0 g	3.00 g/L	Provides group B vitamins that contribute to growth
Glucose anhydrous ($C_6H_{12}O_6$)/Dextrose	5.0 g	4.00 g/L	source of carbon and nitrogen
Calcium chloride dihydrate ($CaCl_2$ x 2 H_2O)	0.25066 g	-	
Magnesium sulphate heptahydrate ($MgSO_4$ x 7 H_2O)	0.5 g	-	
Ammonium sulphate ($(NH_4)_2SO_4$)	0.2 g	-	
Potassium dihydrogen phosphate (KH_2PO_4)	3.0 g	-	
Zinc sulphate monohydrate ($ZnSO_4$ x H_2O)	0.00018 g	-	
Copper sulphate pentahydrate ($CuSO_4$ x 5 H_2O)	0.00016 g	-	
Manganese sulphate hydrate ($MnSO_4$ x 7 H_2O)	0.00015 g	-	
Sodium molybdate dihydrate (Na_2MoO_4 x 2 H_2O)	0.00030 g	-	
Water	1000 ml	1000 ml	

Orange serum	-	5.00 g/L	
Tryptone	-	10.0 g/L	Carbon and nitrogen source
Dipotassium phosphate K_2HPO_4	-	3.00 g/L	Provides osmotic buffer for cell survival
Agar	15-22 g (depending on the gel strength of the agar)	17.0 g	Solidifying agent

Table 45. Extensive ingredient list with their functionalities for PCA and OSA agar, used for the detection of Total Count. The PCA agar is used for the In House-method whilst the OSA agar is used for the IFU-method (Microbiologie Clinique, 2022) (Scharlau Microbiology, 2022).

Ingredients	**PCA**	**OSA**	**Functionality**
Yeast extract	2.5 g/L	3.00 g/L	Provides group B vitamins that contribute to growth
Glucose anhydrous ($C_6H_{12}O_6$)/Dextrose	1.0 g/L	4.00 g/L	Supplementary source of carbon and nitrogen
Water	1000 ml	1000 ml	
Orange serum	-	5.00 g/L	
Tryptone	5.0 g/L	10.0 g/L	Carbon and nitrogen source
Dipotassium phosphate K_2HPO_4	-	3.00 g/L	Provides osmotic buffer for cell survival
Agar	15 g/L	17.0 g	Solidifying agent

Table 46. Extensive ingredient list with their functionalities for YGC and OGY agar, used for the detection of Yeast & Moulds. The YGC agar is used for the In House-method whilst the OGY agar is used for the IFU-method (Sigma Aldrich, 2022) (Corry et. al., 1995) (Condalab, 2022).

Ingredients	YGC	OGY	Functionality
Yeast extract	5.0 g/L	5.0 g	Group B vitamins that contribute to growth, nitrogenous compounds
Glucose anhydrous ($C_6H_{12}O_6$)/Dextrose	20 g/L	20 g	Supplementary source of carbon and nitrogen
Chloramphenicol	0.10 g/L	-	Thermostable antibiotic which provides selectivity
Oxytetracycline	-	0.1 g	Antibiotic which provides selectivity
Agar	15 g/L	12 g	Solidifying agent

Pie charts showing results on Veriflow and ACB-IH method years 2020-2022.

Based on data provided by the Company, an idea of how often the Veriflow method gives a positive and false positive result could be formed. The collected data is from years 2020-2022, with the majority taken from 2022. The results are presented in pie charts in Figure 27 to Figure 29 below.

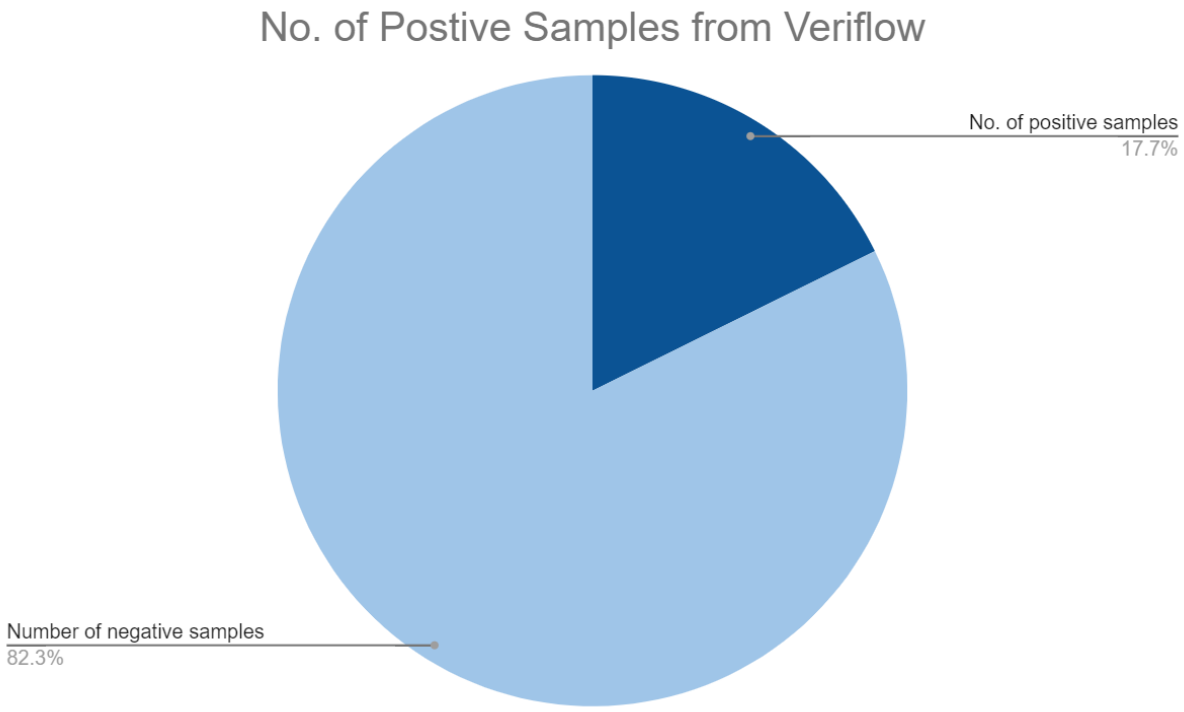

Figure 27. Based on data provided from the Company. Dark blue indicates the number of samples that were positive from the Veriflow test and light blue the number of samples that were negative. Total number of samples tested was 96 from the years 2022-2020.

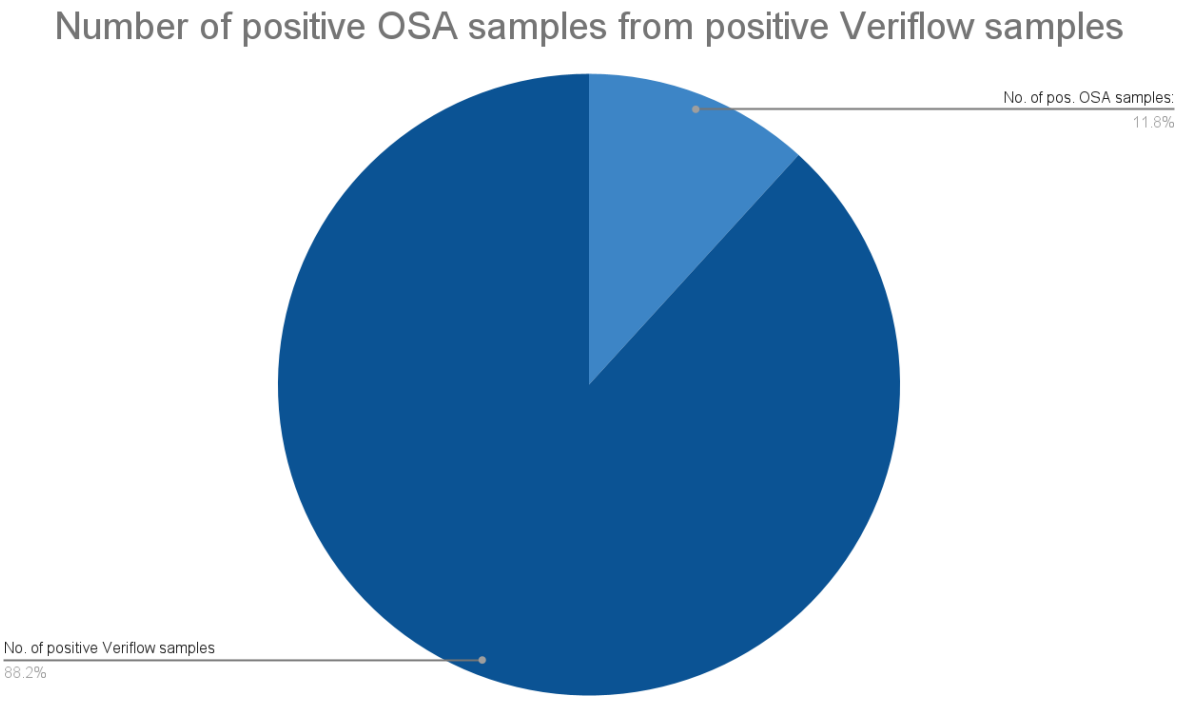

Figure 28. Based on data provided from the Company. Dark blue indicates the number of samples that were positive from the Veriflow test (same as figure 27) and light blue the number of these samples that were negative using the ACB-IH pour plate method with OSA agar.

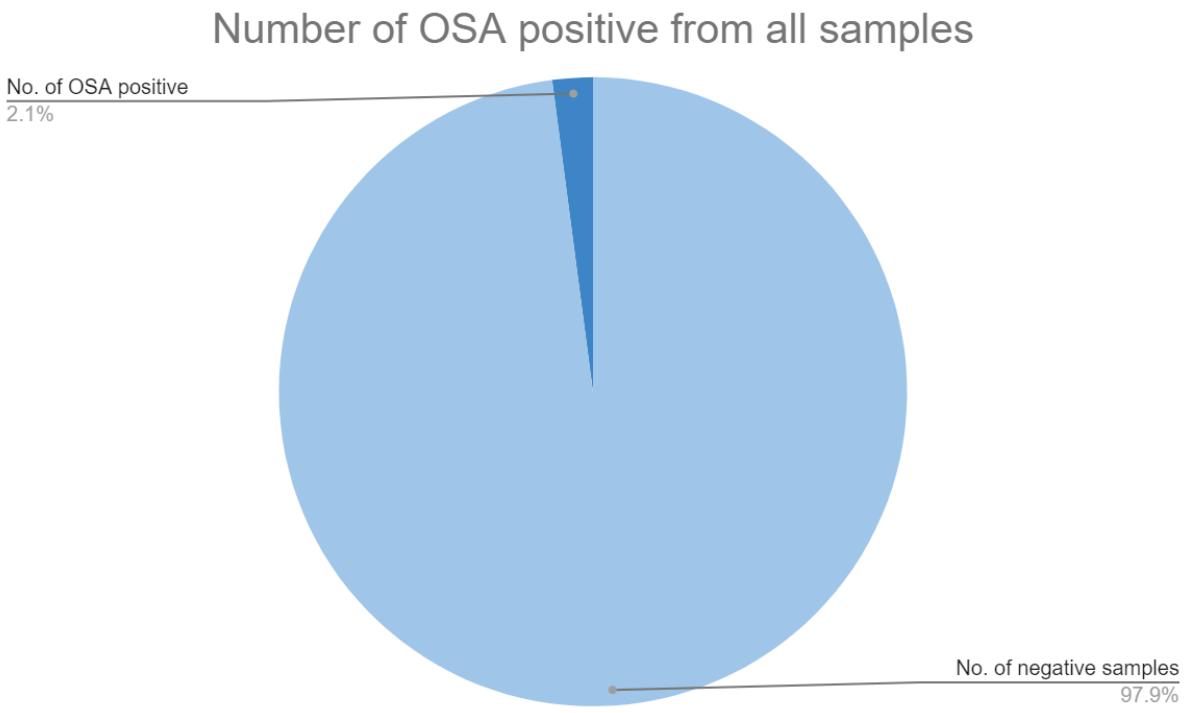

Figure 29. Based on data provided from the Company. Dark blue indicates the number of samples that were positive from the Veriflow test and the ACB-IH method using OSA agar. The light blue is the total number of negative samples (including false positives shown in Figure 28).