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Evaluation of Alternative Analysis Methods for *Salmonella*, Yeast and Mold for Use in Food Business Compared to Reference Methods

Master of Science Thesis

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All featured photographs were taken by the author.

Cover:

The photograph features three different products from B. Engelhardt & Co AB and in the background are plates and supplements for the alternative methods tested as well as plates for the reference methods.

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Abstract

The company B. Engelhardt & Co AB uses conventional culture based methods which are derived from reference methods. While being widely accepted and cheap in consumables these methods often require long or repeated incubation steps. This is particularly true for *Salmonella* detection and yeast and mold enumeration. The aim of the project was to find alternative methods which produced the result faster on these analyses than the current methods while still being accurate, practical and cost-effective.

First information was compiled about different alternative methods and the information was evaluated. After the review of the available methods a selection of two alternative methods for *Salmonella* detection and one alternative method for yeast and mold enumeration were made. The selected methods were Salmonella Precis from Oxoid, IRIS Salmonella from Biokar diagnostics SimPlate Yeast and Mold Color Indicator from Biocontrol systems. These were then investigated experimentally on food products normally analyzed for respective microorganism. Inoculations with pure cultures of *Salmonella* species and related Enterobacteriaceae were also made for the *Salmonella* methods. Tests with both the selected alternative method(s) and the current method were performed and their performance was compared.

From these experiments it could be concluded that IRIS Salmonella offers some advantages over Salmonella Precis. Comparing IRIS Salmonella with the reference method it can be concluded that IRIS Salmonella offers 24 hours faster results while saving operator time. However it is only about 90% accurate relative to the reference and comes at a significantly higher cost. It is up to the quality division at B. Engelhardt & Co AB to decide if this is acceptable or not.

For the SimPlate Yeast and Mold Color Indicator, while it gives a result 48 hours faster than the reference, it was not possible to conclude that the alternative method gives similar results to that of the reference method; significantly different results were obtained. It is also more laborious to use and costs more. The method could not be recommended for use.

Keywords: food quality, alternative methods, *Salmonella*, yeast and mold, detection, enumeration, Salmonella Precis, IRIS Salmonella, SimPlate Yeast and Mold Color Indicator

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1. Introduction

Foods are by its nature often nutritious to microorganisms and often lack the defense mechanisms found in living organisms, leaving foods to be readily spoiled by microorganisms if care is not taken. Microbial spoiling of foods can make it unappetizing for consumers with effects such as textural changes, discoloration, development of off-odors and off-flavors. It can also make the food dangerous if a pathogen such as *Salmonella* is present and allowed to multiply or if toxins are produced. It has been estimated that a quarter of all foods post-harvest and post-slaughter is lost due to microbial spoilage. [Anonymous 1985] Food safety and food quality is therefore a major concern for the food business operator and to ensure safety and consistent quality routine testing is employed.

B. Engelhardt & Co AB is a food company which import, manufacture and distribute powdered food ingredients for the Nordic food industry. Products include nutrition products such as very low calorie diet (VLCD) and high protein, also bakery and pastry products among many others. The company currently uses culture based methods for the microbial quality analyses which are derived from the Nordic Committee on Food Analysis (NMKL) reference methods. The reference methods are internationally recognized and widely accepted methods that are often conventional culture methods. This is due to the conventional culture methods being open access, well accepted and often considered to be the gold standard in food diagnostics. [Jasson, *et al.* 2010] The methods uses selective nutritious broth and agar media to grow, isolate and enumerate the target microorganisms while suppressing the background flora. The conventional culture methods require no expensive laboratory equipment and are cheap in consumables, however they are often require long or repeated incubation steps. This is particularly true for yeast and mold (five days incubation) and *Salmonella* (18h pre-enrichment, 24h enrichment and 24h incubation). It is therefore of interest for B. Engelhardt & Co AB to find alternative rapid methods for these analyses that can be used as a complement to the conventional culture methods in those cases where a shorter analysis time is preferred; for instance if there is a delay from the supplier it may be desirable with a faster analysis in order to deliver the product to the customer within the agreed time. These alternative methods while being faster must be comparable with the classical culture methods in accuracy, be practical to use and cost-effective.

The aim of this thesis was to compile and evaluate rapid alternative methods for qualitative analysis, detection, of *Salmonella* and quantitative analysis, enumeration, of yeast and mold. From this evaluation methods were to be selected and compared experimentally to the reference based methods currently in use at B. Engelhardt & Co AB. The key parameters were time to result, accuracy of the result, cost and practicality.

2. Theory

This chapter aims to provide the reader background knowledge in the relevant microorganisms and how they affect spoilage. It also aims to explain the current methods used and the currently available alternative methods.

2.1. *Salmonella* and salmonellosis

Salmonella is a genus of rod shaped, gram-negative, non-spore forming facultative anaerobes of the family Enterobacteriaceae. They are chemoorganotrophs and most strains are motile with peritrichous (projecting in all directions) flagella. *Salmonella* is an enteric bacterium related to *Escherichia coli* and is normally found in the warm-blooded animal intestine. The genus is mesophilic and has a temperature optimum around 32-37°C but can grow in the wide temperature range 6-46°C. [Odumeru and León-Velarde 2012] It consists of two species, *Salmonella bongori* and *Salmonella enterica*. *Salmonella enterica* can be divided into six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI), most human pathogens are found in subspecies *enterica* (I). [Odumeru and León-Velarde 2012, Madigan 2008] Each subspecies can be divided into serotypes based on their cell surface and flagellar antigens. There are over 2500 serotypes of *Salmonella* and over 1400 of these can cause disease in humans. [Odumeru and León-Velarde 2012, Madigan 2008] Due to this classification the formal name for a *Salmonella* serotype is long and is commonly shortened, for instance the organism *Salmonella enterica* subspecies *enterica* serotype Typhi is typically shortened to *Salmonella* Typhi. Many of the serotypes are named after where they first were isolated such as *Salmonella* Dublin, *Salmonella* London and *Salmonella* Landala among others.

Infection by pathogenic *Salmonella* causes salmonellosis which for most serotypes is characterized by a localized infection of the small and large intestine. Salmonellosis affects about 4000 people in Sweden each year. [Smittskyddsinstitutet 2011] Normal symptoms include watery diarrhea, abdominal pain, nausea, fever and headache. The disease normally resolves itself without antibiotic treatment; but sometimes the patient is given fluid replacement. [Smittskyddsinstitutet 2011] A few serotypes of *Salmonella* can cause septicemia (blood infection) and enteric or typhoid fever. In this form the *Salmonella* pass through the lymphatic system into the blood stream and are carried to various organs. [Volovskaya 1990] This leads to a systemic infection and high fever that lasts for several weeks. Untreated the mortality can reach 15% for this form of salmonellosis, but with antibiotic treatment this rate is reduced to less than 1%. [Madigan 2008]

The ultimate source of *Salmonella* is from the intestine of man and animals. Some serotypes are host-specific but many serotypes can infect both humans and animals; salmonellosis is therefore a zoonotic disease. From the infected host the microorganisms can be released with feces, urine, milk, saliva and nasal discharge. [Volovskaya 1990] *Salmonella* Enteritidis is also known to infect the eggs of poultry. [Dawoud 2011] After infection some humans can become chronic carriers and shed *Salmonella* for many months [Madigan 2008, Volovskaya 1990]. *Salmonella* is stable in the environment and can spread with water, dirt and dust. Since *Salmonella* has to reach the gastrointestinal tract to infect their host it is almost exclusively associated as a foodborne disease but contact infection is possible. [Volovskaya 1990] For healthy adults the infectious dose is up to 100 000 bacteria before symptoms of infection appear but children, elderly and immunocompromised individuals are more susceptible. [Smittskyddsinstitutet 2011] In some cases of fatty foods such as chocolate and cheese the infectious dose seems to be much lower.

[Smittskyddsinstitutet 2011] Due to the high infectious dose for healthy adults the disease is most commonly associated in fresh foods in which *Salmonella* can grow and multiply such as meat, eggs, fish and milk. The most common serotypes associated with foodborne salmonellosis are *Salmonella* Typhimurium and *Salmonella* Enteriditis. [Madigan 2008]

2.2. Yeasts and molds

Yeasts and molds (filamentous fungi) are eukaryotes belonging to the kingdom Fungi. Fungal species that can cause food spoilage is a large and diverse group consisting of hundreds of species and are widely distributed throughout the environment.

Yeasts are predominantly unicellular organisms and can be divided into two categories: facultative anaerobic fermentative yeast and aerobic oxidative yeast. The fermentative yeasts are more common in food spoilage. [Sperber and Doyle 2009] The most common method of reproduction of yeasts is by budding but some species instead divide by binary fission like bacteria. Many species of yeasts can also reproduce sexually by producing spores although this method of reproduction is less common. Yeasts are normally not associated with food infection or poisoning but a few species are opportunistic pathogens and can cause infection in immunocompromised individuals, an example of this is *Candida albicans*. [Madigan 2008] Yeasts can however spoil food and give it unappealing odor and texture. Yeasts generally spoil products with low pH and with sugars, organic acids or other easily metabolized carbon sources. Examples include soft drinks, syrups and salad dressings which are often spoiled by species of *Torulaspora* and *Zygosaccharomyces*, beer and wine can be spoiled by *Saccharomyces* and *Brettanomyces*, while meats and cheeses are susceptible to *Rhodotorula*, *Yarrowia* and *Debaryomyces*. [Kurtzman 2006]

Molds are multicellular filamentous fungi and are composed of a network of thread-like filaments called hyphae. The entire mass or a large portion is called mycelium. Molds are often very hardy and can grow across a broad spectrum of temperatures. Certain molds can grow in exceptionally harsh and can spoil foods that would otherwise be microbiologically stable. Some molds can produce mycotoxins which are harmful to humans. Common food spoilage genera include: *Penicillium*, *Aspergillus*, *Rhizopus* and *Fusarium*. [Sperber and Doyle 2009]

2.2.1. Factors which affect spoilage by yeasts or molds

In general bacteria multiply at a faster rate than yeasts and molds and generally faster growing microorganisms have an advantage over slower growing microorganisms. However molds and yeasts have different characteristics that permit them to dominate in environment where the physico-chemical factors cause bacteria to grow very slowly or not at all. The following paragraphs will describe some of these factors.

Water activity (a_w) is based on Raoult's Law for ideal solutions and does not take into account solute interactions with components other than the solvent; hence it is as most accurate for dilute solutions. Water activity is defined as $a_w = p/p_0$ where p is the partial pressure of water vapor in the material and p_0 is the saturation vapor pressure of pure water under the same conditions. It is a measurement of the freely available water in the food material. Pure water has a water activity of 1 and all other solutions have a water activity of less than 1. Most microorganisms cannot grow when the water activity is below 0.90, including the vast majority of human pathogens. [Grant 2004] Most fresh foods have a_w values in the range 0.99 – 0.95 which allow growth for most microorganisms. [Grant 2004] Lowering the water activity below this range prevents growth of many organisms and

retards spoilage. As the water activity drops the microbial cell is exposed to a higher external osmotic pressure and unless the cell can balance this it will become dehydrated which eventually will lead to inability of growth and even death. The only prokaryotes able to thrive at a_w below 0.8 are the haloarchaea; however they can only spoil heavily salted proteinaceous foods. [Grant 2004] Yeasts and mold are often outcompeted by the fast growing prokaryotes at high a_w values but for foods with a water activity in the lower range, such as for confectionary and jams, yeasts and molds usually dominate. The most exceptional microorganism with the lowest requirement for available water currently described is the spoilage mold *Xeromyces bisporus* which can grow at a water activity of 0.61. [Grant 2004, Pitt and Hocking 2009] However the majority of molds and yeast are inhibited by a_w values between 0.8 – 0.75. [Grant 2004]

Another important factor in food spoilage by fungi is pH. It is defined as: $pH = -\log_{10} [H^+] = \log_{10} 1/[H^+]$ where $[H^+]$ is the hydrogen ion concentration. Most bacteria thrive near neutral pH with only few being able to grow below a pH of 4.5 or above a pH of 9. [Sperber and Doyle 2009] As pH decreases bacteria are progressively inhibited which opens up for fungi to colonize as they are little affected by pH in the range 3-8. [Pitt and Hocking 2009] A few fungal species are capable of growing at pH 2 and below. [Pitt and Hocking 2009] Weak acids can be used as preservatives as these can enter the microbial cell in its uncharged undissociated form and dissociate once it enters the relative higher pH environment of the cell and cause a lowering of pH inside the cell. The organic acid ions can also have an antimicrobial effect. [Betts 2006] Certain fungal species possess resistance to the action of weak acids and can grow and spoil foods containing these preservatives.

Temperature directly affects the rate of biochemical reactions and thus the growth rate of microorganisms. Low temperatures have a preservative effect not only by slowing biochemical reactions but also by introducing negative internal cellular changes. [Sperber and Doyle 2009] While refrigerated foods spoil less readily a few fungal species have developed mechanisms enabling them to grow even at subzero temperatures down to -7°C. [Pitt and Hocking 2009] Ice crystal formation during freezing can kill a portion of the microbial population but it will not destroy the entire population and in fact freezing is a common method to preserve microbial cultures. [Sperber and Doyle 2009]

Molds are obligate aerobes and can only grow in the presence of oxygen. Complete removal of all oxygen is however almost impossible to accomplish and some molds are effective scavengers of oxygen and can grow at oxygen levels as low as 0.4%. [Sperber and Doyle 2009] Even in an oxygen free environment food spoilage can occur from anaerobic bacteria and fermentative yeasts.

2.3. Current methods

This section will outline the methods currently in use by B. Engelhardt & Co AB and these methods will be used as the reference in all experiments.

2.3.1. Culture method for *Salmonella* detection

The first step in the Salmonella protocol is the pre-enrichment step. During the processing of food the microorganisms present may be damaged, either fatally or sub-lethally. These sub-lethally injured microorganisms can resuscitate and become functionally again with the right conditions. However, depending on the nature of injury sustained, the sub-lethally injured microorganisms may not grow well in selective media. This can, for instance, be due to damages in the cell membrane that affects permeability and makes the cell susceptible selective agents or antimicrobials. In order to

make sure there are no sub-lethally injured target microorganisms present in a sample it is necessary to have a resuscitation step, often called a pre-enrichment step, to give cells the conditions and time to repair their damages and return to a normal physiological state and regain their normal resistance to the selective agents. This is important to take into account when screening for *Salmonella* where there are not allowed for any potentially viable *Salmonella*. Therefore most conventional and alternative methods incorporate a pre-enrichment step to resuscitate sub-lethally injured cells. [Wu 2008]

For the first pre-enrichment step the sample of 25g is placed in a stomacher bag and diluted in 225 ml buffered peptone water to a 1:9 sample/broth ratio. This sample is homogenized in a stomacher machine (laboratory paddle blender) and pre-enriched in an incubator for 18 hours at 37°C. For the next step, the selective enrichment step, 0.1 ml of the pre-enriched sample is transferred to a glass tube containing 10 ml Rappaport-Vassiliadis broth and mixed in a vortex. Rappaport-Vassiliadis broth was first formulated by Rappaport, F., Konforti, N. and Navon, B. (1956) when they found a combination of magnesium chloride and malachite green to be selective for *Salmonella*. It was later modified by Vassiliadis, P. *et al.* (1978) by reducing the malachite green concentration to make it suitable for incubation at 43°C which improved the selectivity. Rappaport, F., Konforti, N. and Navon, B. (1956) also noted that malachite green is toxic to *Salmonella* Typhi and thus Rappaport-Vassiliadis broth is not suitable to use for that serotype. The enrichment sample is incubated for 24 hours at 41.5°C. After the enrichment step the sample is streak on a pre-poured solidified XLD-agar plate by a loop (10µl) in a manner that isolated colonies can be acquired.

XLD-agar (Xylose-Lysine-Desoxycholate Agar) is used to identify both *Shigella* species and *Salmonella* species from non-pathogenic enteric bacteria. It has a pH of 7.4 and contains the pH indicator phenol red which at this pH it appears bright red. *Shigella* is one of few enteric bacteria unable to ferment xylose. Other enteric bacteria, including *Salmonella*, ferment the added xylose which lowers the pH and the indicator color changes to yellow around these colonies. After the *Salmonella* have used up the xylose it will decarboxylate the lysine present which increases the pH once again leaving the colonies bright red as the *Shigella* and separates them from enteric bacteria with xylose activity that lack lysine decarboxylase. *Salmonella* then metabolize the sodium thiosulphate which is complex bound to ferric ammonium citrate. As the thiosulphate are metabolized to hydrogen sulfide the ferric ammonium citrate will precipitate and produce black centers in the *Salmonella* colonies allowing differentiation from *Shigella* colonies. Sodium desoxycholate is added to the medium to inhibit gram-positive bacteria. Typical *Salmonella* colonies are pink with or without black centers. A few atypical *Salmonella* produce yellow colonies with or without black centers. [Andrews, W. H., Jacobson, A. and Hammack, T. 2011] The plates are incubated at 37°C for 24 hours and are then read. Figure 1 shows a typically *Salmonella* species (*Salmonella* Enteritidis) growing on an XLD-agar plate, with the characteristic red colonies and black centers.

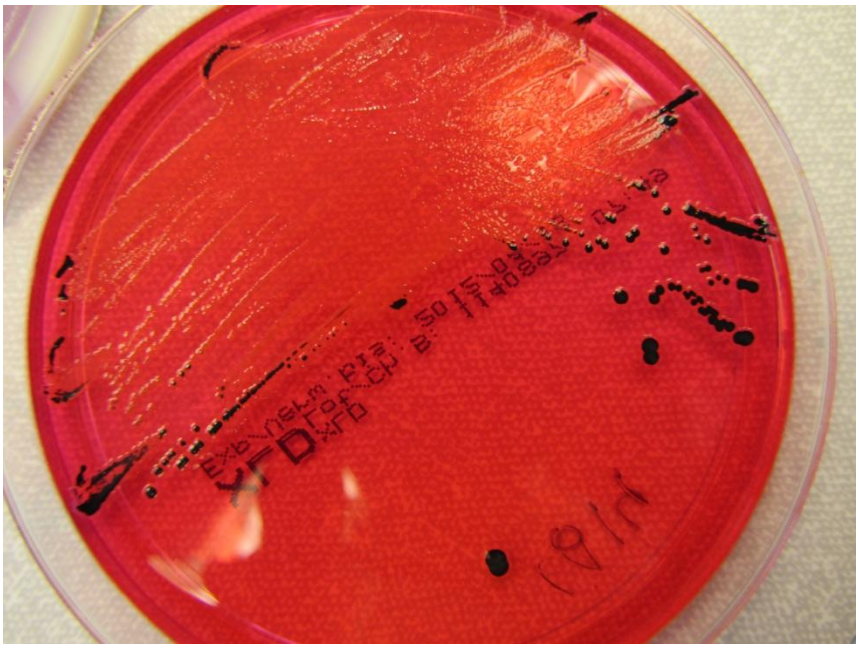

Figure 1: *Salmonella* Enteritidis growing on a XLD-agar plate.

2.3.2. Culture method to enumerate yeasts and mold in foods

The culture method to enumerate yeast and molds is quite direct. The sample is placed in a stomacher bag and diluted in 0.1% peptone water solution to achieve a 1:9 sample/broth ratio. The diluted sample is then homogenized in a stomacher machine for 30 seconds. This can then be further diluted in peptone water and mixed to achieve higher dilutions. From each dilution the sample is transferred by pipette to a pre-poured solidified Yeast Extract Glucose Chloramphenicol (YGC) agar and the inoculums are spread with a sterile, bent glass rod. YGC-agar is a complex media and contains as the name implies yeast extract, glucose and chloramphenicol, which is a broad spectrum antibiotic to suppress bacterial growth. All plates are inoculated for five days at 25°C and then counted. Results are reported as CFU (colony forming units) yeast/g and CFU mold/g or CFU fungal species/g. Figure 2 shows mold colonies growing on several YGC plates stacked on top of each other.

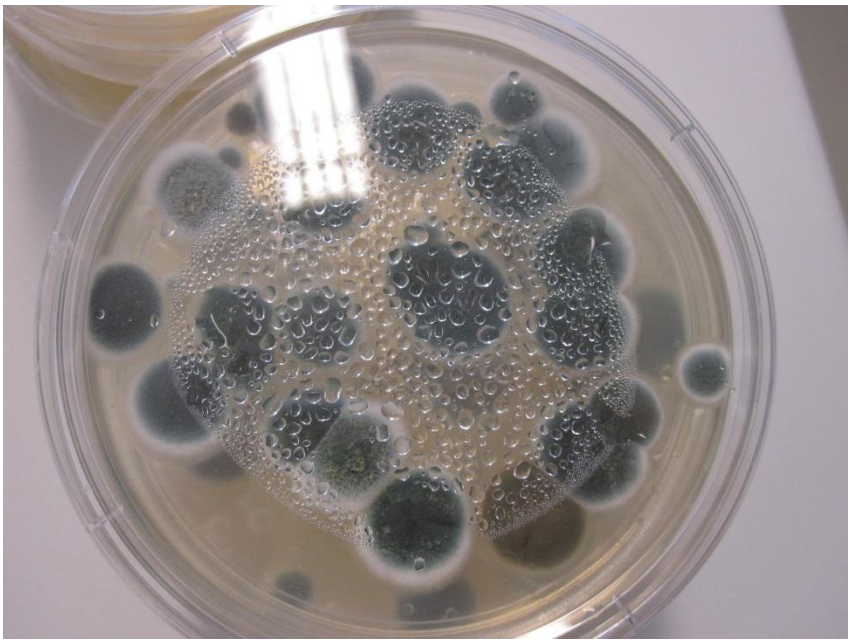
Figure 2: Mold colonies on YGC plates.

2.4. Alternative methods

An alternative method is defined in EN/ISO (International Organization for Standardization) standard 16140 (2009) method validation as a "method of analysis for detection or estimation, of the same analyte as is measured by using the corresponding reference method, for a given category of products." The alternative method can address different undesired characteristics of the reference method such as speed of analysis, analytical properties, cost and ease of execution. The available alternative methods can be divided into two broad categories; alternative methods for enumeration and alternative methods for detection.

2.4.1. Alternative methods for enumeration

The available alternative methods for enumeration can be further divided into several categories: modified culture methods, biochemically based methods and microscopic based methods. [Jasson, *et al.* 2010]

2.4.1.1. Modified culture methods

Modified culture methods are usually based either on colony count like the reference methods or are Most Probable Number (MPN) estimations. Two examples of colony count alternative methods are Petrifilm™ (3M™) and Compact Dry (Nissui Pharmaceutical Co., LTD.). In the reference method the media is contained in a Petri dish but in both these methods it is instead distributed across a thin plastic film which contains chromogenic enzyme substrates aimed to ease the differentiation of yeasts from molds. Due to their smaller size they occupy less space in the incubator. Both manufacturers specify a range for incubation for yeast and mold enumeration (3-5 days for Petrifilm™ and 3-7 days for Compact Dry) however both methods rely on the microorganisms to form colonies visible to the human eye albeit with the help of chromogenic agents. [3M 2012, Nissui Pharmaceutical Co., LTD. 2010]

Biocontrol systems' product SimPlate® is a modified culture method that offers detection and enumeration without the need for visible colonies. Instead it uses Binary Detection Technology™ to detect and enumerate. For the yeast and mold method the sample is mixed with reconstituted liquid culture media and poured unto the SimPlate device consisting of 84 wells and a sponge to drain away excess liquid after all wells are filled. The device is incubated for three days. As the fungal enzymes react with the substrates in the media it will produce a color change and after the incubation period the wells that exhibit a color change will be counted and converted to MPN using the table provided with the product. The counting range is from <10 to 7380 in the lowest dilution specified in the protocol which means one SimPlate device can replace two Petri dishes. The method can give a result faster than the reference method due to the fewer number of microorganisms needed to produce a color change than to form a visible colony on an agar plate. [Biocontrol systems 2012]

The Soleris™ technology from Neogen also uses color change in the media to enumerate growth by detecting pH change and other biochemical reactions. The sample is inoculated into a vial containing liquid growth media and other reagents and is read at predetermined time intervals photometrically by a machine. For yeast and mold enumeration the required time to a result are 72 hours. [Neogen 2012]

For large scale analysis there are automated methods like Bio-Mérieux TEMPO® which uses an automated MPN enumeration method. The method uses a card with 3 sets of 16 wells with a 10 fold difference in volume between each well. The sample is mixed with the media which contain

fluorescent indicators. The card with the wells is incubated for three days for yeast and mold enumeration and then read in the TEMPO® Reader which counts fluorescent wells and calculates the MPN. [Bio-Mérieux Industry 2012]

2.4.1.2. Biochemically based enumeration

When microorganisms grow their metabolism can produce charged end-products from uncharged or weakly charged substrates in the growth media. This increases the conductivity of the media and change the impedance variation of the media which can be read in regular intervals. The time required to reach a point where growth is first detected is inversely proportional to the initial microbial population and is constant for any organism growing under defined test conditions. Some organisms, particularly yeasts and molds do not produce strongly ionized metabolites and do not result in large changes in impedance when growing. It is therefore important to read the net change in impedance. However it is critical that the conditions are constant and that the population's generation time is more or less constant. The BacTrac system from Sylab is an example of an impedance-based system which has a special media for analyzing yeast and molds. [Sylab 2012, Silley and Forsythe 1996]

2.4.1.3. Microscopic based enumeration

Microscopic based methods include flow cytometry, which counts microorganisms that pass a light beam by detecting the scatter and fluorescence, and Direct Epiflourescent Filter Technique (DEFT) which concentrate cells from a sample on a polycarbonate membrane filter where they are dyed and then counted under epifluprescence. Both of these methods are designed for fluid products such as water, beverages and milk rather than solid samples and are therefore of little interest in this project.

2.4.2. Alternative methods for detection

Similarly for detection methods there are several categories: modified culture methods, enzyme linked immunosorbent assay (ELISA), enzyme linked fluorescent assay (ELFA), lateral flow devices, immunomagnetic separation and concentration, bacteriophage-based detection methods and polymerase chain reaction (PCR). [Jasson, *et al.* 2010]

2.4.2.1. Modified culture methods

A common approach to modify the conventional culture methods is to combine the pre-enrichment and the enrichment steps into a one-step enrichment that enables both resuscitation of sub-lethally injured *Salmonella* and selective enrichment, saving one day of incubation. It is reasonable to assume that due to the one-step enrichment broth needs to be able to resuscitate sub-lethally damaged cells as well as to be selective; it is not as selective as the Rappaport-Vassiliadis broth. This is combined with a chromogenic agar with selective agents to help differentiate and identify *Salmonella*. Examples include Oxoid's Salmonella Precis and IRIS Salmonella from Biokar diagnostics. [Oxoid 2012]

Another approach is to inoculate the pre-enriched sample directly to a Petri dish with semi-solid media and selective agents that allow motile strains of *Salmonella* to move away from the inoculation point to be differentiated and identified. This method will also save one day of incubation due to the enrichment step is combined with the culture step. These tests are designed to only detect motile strains of *Salmonella*. Examples include SESAME Salmonella from Biokar diagnostics and AES CHEMUNEX's Simple Method Salmonella (SMS). [Biokar diagnostics 2012]

2.4.2.2. Immunoassays and bacteriophage based methods

Immunoassays use the specificity of antibodies towards their antigen to identify the target microorganism. The detection limit is approximately 10^4-10^5 cfu/ml and hence enrichment is often necessary. [Jasson, *et al.* 2010]

Most alternative method immunoassays are enzyme linked immunosorbent assay (ELISA) of the sandwich type. The method uses antibodies specific to an antigen of the target microorganism. These antibodies are affixed to a surface of a well, often a microtiter plate. The sample is added and the target organisms bind to the antibodies. The unbound content is removed in a washing step and antibodies that also bind specifically to the target are added and allowed to bind. Enzyme linked secondary antibodies are added that binds to the previously added antibodies and all unbound antibodies are washed away. The enzyme linked to the secondary antibodies convert added substrate to a detectible signal. The detection takes about 2-3 hours and therefore tests are available that only take about one day with the enrichment and analysis. Many ELISA tests are available as automated analysis systems. A variation of the ELISA is the enzyme linked fluorescent assay (ELFA) in which the chromogenic substrates often used in ELISA are replaced by fluorescence for end point detection. An example of this is the VIDAS® system by Bio-Mérieux which is an automated ELFA system. [Jasson, *et al.* 2010]

Lateral flow device (LFD) methods are designed to be a fast and simple detection test for a target substance or microorganism and are most commonly known for their use in pregnancy tests which often are lateral flow devices. The sample is applied to the prefabricated test strip and flows through a porous membrane by capillary action. The membranes are often attached to a plastic housing to protect the test and give it robustness. Along the way in the membrane the sample will encounter a release pad with labelled antibodies specific to the target that will bind to the target if present. These antibodies are labelled with coloured or fluorescent nanoparticles to minimize obstruction of the flow through the membrane. The sample mixed with labelled antibodies will continue to flow along the membrane and encounter a test line where immobilized antibodies specific to the target will bind the target and form an antibody-antigen-antibody sandwich and a visual line will show on the strip indicating detection of the target. Further downstream is a control line located which have immobilized antibodies specific to the labelled antibodies and a response on the control line will indicate flow to the test strip and release of the labelled antibodies. At the end of the test strip is an absorbent pad to wick the liquid and help maintain the flow. [Posthuma-Trumpie, Korf and van Amerongen 2009] SDIX sells a method called RapidChek® SELECT™ Salmonella which uses one-step enrichment together with a lateral flow device test to detect *Salmonella* species. The LFD test itself only takes 10 minutes and the whole protocol can be completed in about one day. This particular method also uses bacteriophages to attack and reduce competitive and cross-reactive bacteria during enrichment. [SDIX 2012]

Immunomagnetic separation and concentration (IMS) is a method that can be used to replace the selective enrichment step in *Salmonella* detection methods and potentially reduce the protocol by about 24 hours. The method is utilizing superparamagnetic particles coated with specific antibodies to capture target cells in a sample and then isolate those using magnets which will trap the coated particles. There are commercially available monosized superparamagnetic polymer particles called "Dynabeads" for the selective isolation of *Salmonella*. [Jasson, *et al.* 2010, Jeniková, Pazlarová and Demnerová 2000]

Instead of using antibodies to capture target microorganisms the specificity of bacteriophages can be used. Bacteriophages are virus that target bacteria and are exceptionally host-specific and this can be used in combination with paramagnetic particles like IMS by coating them with specific phage derived proteins instead of antibodies. Bacteriophages can also be used to attack and lyse target cells to release their enzymes. One described method uses IMS to concentrate the target cells and uses bacteriophages to release adenylate kinase from the target microorganism. With the addition of ADP the akenylate kinase converts it to ATP and this can be detected by firefly luciferase. [Hagens and Loessner 2007]

2.4.2.3. Molecular based methods

One of the most well-known molecular based methods for detection is the polymerase chain reaction (PCR). It is a technique to amplify a strand of DNA first described by Mullins and Faloona (1987). The PCR technique contains three steps that are cycled. It starts with the high temperature denaturation step in which the double stranded DNA in the sample is denaturated to create two complimentary single stranded DNA molecules. The temperature is lowered in the next annealing step where primers, short oligonucleotides whose sequence matches the end of the region of interest, bind to the single stranded DNA molecules. The third and last step is the elongation step in which a heat stable DNA polymerase is activated and synthesize the complimentary strand of the DNA molecule the primer is bound to, creating double stranded DNA. In the subsequent cycles the primers can bind and synthesize complimentary strands to both the original DNA and the newly synthesized strands from the previous steps resulting in an exponential increase in the number of copies. This technique can be used in the detection of *Salmonella* by amplifying DNA sequences unique to *Salmonella* if present. While the PCR analysis is fast the samples are often enriched by incubating from 6-24 hours before the cells are lysed and run in the PCR. Several commercially available automated PCR systems for *Salmonella* exist that provide next day result. An example of this is DuPont Qualicon BAX® System. [Jasson, *et al.* 2010, DuPont 2012] PCR can also be performed manually but this requires too long handling times to be practical for routine analysis.

3. Selection of methods

This section will deal with the process of selecting methods.

3.1. Selection criteria

The main aim of this project was to find and evaluate alternative methods to have a complementary method to use when a faster analysis is desirable. Therefore only methods that provide a shorter time to analysis than the current reference methods were of interest. The test results obtained from the alternative methods should also be accurate when compared to the reference method, as it is no point in a fast result if it cannot be trusted. It is also important that the preparations and handling time do not excessively occupy the laboratory personnel's time. Due to the alternative methods are primarily to be used as complements to the reference method it is also important the methods does not require expensive lab equipment not currently present in the laboratory at Engelhardt & Co. However it was allowed to be more expensive than the reference method.

As this study contains alternative methods for the analysis of *Salmonella* it was important to take into account current legislation when considering alternative methods. According to EU regulation 2073/2005 “on microbiological criteria for foodstuffs” it states that food business operators may use

alternative analytical methods if the methods are validated. For a proprietary method it has to be certified by a third party in accordance with the protocol set out in EN/ISO standard 16140 or other internationally accepted similar protocols. Yeast and mold counts used to be regulated in a similar matter but the current regulation does not contain any criteria for yeast and mold counts. It is still however used by food business operators as an indication of product quality. Salmonella species has to be absent in the sample. The threshold levels for yeast and mold counts are up to the food business operator to agree upon with their customers.

EN/ISO standard 16140 is a technical protocol for the validation of alternative methods for the microbiologic analysis of food and animal feeding stuffs. The performance indicators are inclusivity, exclusivity, relative accuracy, relative specificity, relative sensitivity and detection limit. These are all compared to the corresponding ISO method, which acts as the reference, for the same function as the alternative method. The ISO methods are generally very similar to the NMKL methods and this is true for the *Salmonella* and the yeast and mold method. Inclusivity tests for the alternative method's ability to detect the target microorganism from a wide range of strains and the exclusivity tests for the lack of interference from a relevant range of non-target microorganisms.

The relative accuracy is the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples and is calculated from this equation:

$$Relative\ accuracy = \frac{PA + NA}{N}$$

Where PA is positive agreement where both methods show positive, NA is the negative agreement where both methods show negative and N is the number of tests.

The relative specificity is the ability of the alternative method to not detect the target microorganism when it is not detected by the reference method and is calculated from this equation:

$$Relative\ sensitivity = \frac{PA}{PA + ND}$$

Where ND is the negative deviation where the reference method show positive but the alternative method show negative.

The relative sensitivity is the ability of the alternative method to detect the target when it is detected by the reference method and is calculated from this equation:

$$Relative\ specificity = \frac{NA}{NA + PD}$$

Where PD is the positive deviation where the alternative method show positive but the reference show negative.

These values are calculated from the performance of both methods, alternative and reference, on a range of test samples which are both naturally and artificially contaminated as well as uncontaminated.

The relative detection limit tests the level of contamination for which less than 50% of the responses obtained are positive and that for which more than 50% of the responses obtained are positive.

One of the criteria of the alternative methods considered in the thesis was that it was accurate. Therefore only methods that are validated according to ISO 16140 are considered in this thesis. This is especially important for *Salmonella* methods as they need to comply with EU law. This narrows down the list of potential candidates. There are three major third party agencies that provide validation for alternative methods according to the ISO protocol: MicroVal, NordVal and AFNOR. [Jasson, *et al.* 2010] The American validation agency AOAC does not follow ISO standards and are therefore not considered in this thesis, however information whether the method has also been validated by AOAC will be added in the tables.

Below is table 1 with a list of all alternative *Salmonella* detection methods validated by at least one of the three agencies:

Table 1: List of validated *Salmonella* methods (as of 2012-03-14).

Test kit	**Validation(s)**	**Type**
IBISA	AFNOR	Culture
IRIS Salmonella	AFNOR	Culture
RAPID' Salmonella	AFNOR NordVal AOAC-RI	Culture
Salmonella Precis	AFNOR AOAC-RI	Culture
SESAME Salmonella Test	AFNOR	Culture
Simple Method For Salmonella (SMS)	AFNOR	Culture
RIDASCREEN Salmonella	AFNOR MicroVal	ELISA
Salmonella OPTIMA	AFNOR AOAC-RI NordVal	ELISA
Salmonella SELECTA	AFNOR NordVal	ELISA
TAG 24 Salmonella	AFNOR	ELISA
Tecra Unique Salmonella	AFNOR	ELISA
Transia Plate Salmonella Gold	AFNOR AOAC-RI NordVal	ELISA
VIDAS Easy Salmonella	AFNOR	ELFA
VIDAS ICS2-Plate	AFNOR	ELFA
VIDAS ICS2-SLM	AFNOR	ELFA
VIDAS Salmonella	AFNOR AOAC-RI	ELFA
VIDAS Salmonella Xpress	AFNOR	ELFA
VIDAS Up Salmonella	AFNOR AOAC-RI	ELFA
Oxoid Salmonella rapid test	AFNOR AOAC-RI	Immunological
RapidChek SELECT Salmonella	AFNOR AOAC-RI	LFD

Reveal Salmonella 2.0	AFNOR	LFD
Lumiprobe 24 Salmonella	AFNOR	Molecular hybridization
ADIAFOOD Salmonella	AFNOR AOAC-RI	PCR
Assurance GDS Salmonella	AFNOR AOAC-RI	PCR
BAX Salmonella PCR	AFNOR AOAC-RI NordVal	PCR
Biotest MMB Salmonella method	MicroVal	PCR
foodproof Salmonella Detection Kit	AOAC-RI NordVal	PCR
GeneDisc Salmonella spp.	AFNOR	PCR
HQS Salmonella	AFNOR	PCR
IQ-Check Salmonella II	AFNOR AOAC-RI NordVal	PCR
MicroSEQ Salmonella *spp.* Detection Kit	AFNOR AOAC-RI	PCR
SureFood PATHOGEN Salmonella PLUS	AOAC-RI MicroVal	PCR
TAQMAN Salmonella	AFNOR	PCR

Below is a table (table 2) of alternative yeast and mold enumeration methods validated by at least one agency:

Table 2: List of validated yeast and mold methods (as of 2012-03-14).

Test kit name	Validation	Type	Time
Compact Dry YM	AOAC MicroVal NordVal	Culture	5 days
3M Petrifilm Yeast and Mould Count Plate	NordVal	Culture	5 days
SimPlate Yeast & Mold Color Indicator	MicroVal	BDT	3 days

As a part of the project three external laboratories (ALControl, Eurofins and ALS) which housed validated alternative methods based on PCR, ELISA or ELFA were contacted to investigate the potential option to have samples tested by them but due to the shipping time they could not offer a faster analysis than the culture based methods. The price ranged from 678 SEK to 800 SEK with minimum 48 hours to a result. None of the contacted laboratories could offer rapid yeast and mold analysis.

3.2. Selection of methods for *Salmonella*

As the selected methods are primarily going to be used as a complement to the existing NMKL based methods one of the criteria was that it would not require acquisition of expensive lab equipment and therefore all methods that does require that are excluded. This excludes all PCR, ELISA and ELFA based methods and the molecular hybridization method as they require the purchase of a specific

machine (table 1). The external laboratories were also excluded due to the high cost per test with only 24 hours faster result.

Below is a more detailed table (table 3) of the validated alternative *Salmonella* methods which do not require purchase of expensive lab equipment. In this stage more information were gathered for each alternative method. Two distributors (VWR International AB and Oxoid AB) failed to answer some detailed questions regarding the method which they are selling within the time limit. Distributors had several weeks to supply this information and were also notified that there was a time limit to when the information had to be provided. The missing information is indicated by a question mark. Positive properties are marked in green while negative properties are marked in red. All performance data were acquired from the respective AFNOR validation reports and the other data were provided from respective distributor as noted in the table.

Table 3: Detailed list of potential candidates for *Salmonella* detection.

Alternative method	Validation	TTR	Size	Maximum shelf-life[d]	Rel. A; Sp; Se (%)	Detection limit (CFU/25g)	Price/test	Type	Storage conditions	Inclusivity	Exclusivity	Distributor
RapidChek SELECT Salmonella	AFNOR AOAC-RI	1 day	100 tests	1 year	95,3; 97,0; 93,3	0,5-2,2	64 SEK	LFD	RT and RF	43/51	0/30	SDIX Europe Ltd, Hampshire, UK
IBISA	AFNOR	2 days	20 plates	?	91,9; 93,8; 89,7	0,3-2,2	?	Culture	RF	53/53	2/30	VWR International AB, Stockholm, Sweden
Simple Method Salmonella (SMS)	AFNOR	2 days	?	?	99,0; 99,0; 98,0	0,151-0,703	?	Culture	RF	57/64[a]	0/30	VWR International AB, Stockholm, Sweden
Reveal 2.0	AFNOR AOAC-RI	2 days	20 tests	6 months	90,8; 89,9; 91,7	0,3-1,6	131.75 SEK + Freight	LFD	RT	51/56	0/30	ANL Produkter AB, Älvsjö, Sweden
Oxoid Salmonella Precis	AFNOR AOAC-RI	2 days	20/120 plates	11 weeks	91,0; 92,0; 89,9	0,1-1,8	47,6 - 55,6 SEK[bc] + Freight	Culture	RF	52/53	2/40	Oxoid AB, Malmö, Sweden
RAPID' Salmonella	AFNOR AOAC-RI NordVal	2 days	20 plates	90 days	91,2; 92,4; 89,8	0,1-1,8	26,25 SEK + Freight	Culture	RF	47/51	13/42	Food Diagnostics AB, Gothenburg, Sweden
Oxoid Salmonella rapid test	AFNOR AOAC-RI	2 days	50 vessels	457 days	97,3; 96,2; 98,6	0,1-1,8	?	Immuno logical	RT and RF	52/55[a]	0/30	Oxoid AB, Malmö, Sweden
SESAME Salmonella	AFNOR	2 days	20/120 plates	90 days	97,0; 97,1; 96,9	0,1-1,2	23,6 – 48 SEK[b]	Culture	RF	62/62[a]	0/35	Biolab A/S, Risskkov, Denmark
IRIS Salmonella	AFNOR	2 days	20/120 plates	75 days	90,0; 88,0; 91,6	0,2-2,0	26,2 – 50 SEK[b]	Culture	RF	58/58	0/30	Biolab A/S, Risskkov, Denmark

[a] – Can only detect motile strains

[b] – Price range: 120 plates – 20 plates

[c] – Discounts included in price

[d] –For the component with the shortest

Abbreviations: Rel. A; Sp; Se (%) – relative accuracy, relative specificity and relative sensitivity.
TTR – time to result
RT – Room temperature
RF – Refrigeration

Looking at table 3 some methods could be excluded directly. The methods with incomplete information will not be considered. The methods with incomplete information were IBISA, Simple Method Salmonella (SMS) and Oxoid Salmonella rapid test. Some methods, marked with a superscript a on the table, are only designed to detect motile strains of *Salmonella* as they use this ability in the selection. Due to this limitation in inclusivity these method were also excluded. The two LFD methods (RapidChek® SELECT™ Salmonella and Reveal 2.0) are also limited in their inclusivity due to the limited space and the large variation in the *Salmonella* genus. For instance according to SDIX Europe Ltd their product RapidChek® SELECT™ Salmonella cannot detect any *Salmonella* from the serotype group 18 (with *Salmonella* Cerro, *Salmonella* Carnac and *Salmonella* Toulon, also called group K) due to the limited space, even though they have antibodies for all serotype groups. So due to their inherent limitation in inclusivity these methods were also excluded.

This leaves the three modified culture methods Salmonella Precis, RAPID' Salmonella and IRIS Salmonella. RAPID' Salmonella has been reported by the NordVal report to have a high number of false positives and this can also be seen on the high number of cross-reactions (exclusivity) in the AFNOR validation report and was therefore excluded due to the extra work this would generate as many results would need confirmation. [NMKL 2012]

3.2.1. Selected methods for *Salmonella*

This leaves Salmonella Precis and IRIS Salmonella. Both have very similar scores with IRIS Salmonella performing better at the inclusivity and exclusivity tests but Salmonella Precis scoring better at the relative accuracy and relative specificity. IRIS Salmonella is slightly less expensive at the 20 plate package but gets considerably cheaper if bought in larger packages of 120 plates due to the high freight charge from Denmark gets divided on 120 plates rather than 20 plates. For Salmonella Precis the freight cost is not included in the price as B. Engelhardt & Co AB regularly purchases supplies from Oxoid AB and therefore gets free shipping as well as discounts on the price. The discount is included in the price for Oxoid AB's products. The maximum shelf lives are similar for both plates. Due to them being so similar both were selected for testing to see which one performed best at the conditions and products at B. Engelhardt & Co AB. Both methods use a one-step enrichment which is made from dissolving dehydrated media in distilled water, autoclaving the media and then add a supplement. However the method of supplement application differs. Salmonella Precis uses a freeze dried pellet which has to be dissolved in sterile water before added to the liquid media while IRIS Salmonella uses a tablet in a blister pack which simply has to be added to the solution. The supplement tablet also contains coloration agents which turns the supplemented liquid media green. Both plates use an opacifier to make the plate medium opaque and chromogenic substrates to ease the identification and differentiation of colonies. Figure 3 and figure 4 shows the reaction of a typical Salmonella serotype (*Salmonella* Enteritidis) when growing on IRIS Salmonella (figure 3) and Salmonella Precis (figure 4) respectively.

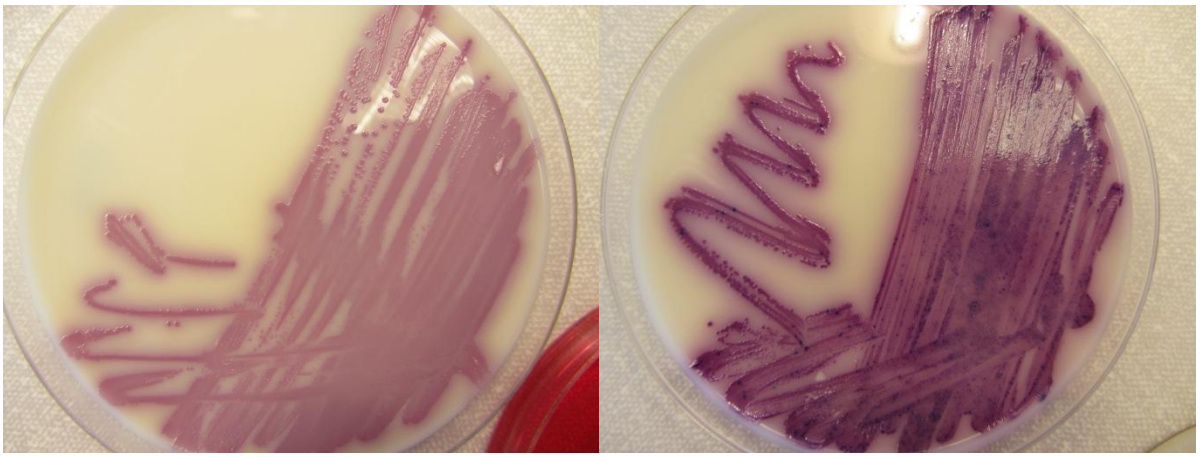

Figure 3 (left) and figure 4 (right): *Salmonella* Enteritidis growing on an IRIS Salmonella plate (left) and on a Salmonella Precis plate (right).

3.3. Selection of methods for yeast and mold

The current reference method for yeast and mold enumeration requires 5 days for a result looking at table 2 there is only one method that offers a validated faster result than the reference. While the other two methods offer some advantages they do not offer a shorter time to detection and is thus of no interest in this thesis.

3.3.1. Selected method for yeast and mold

SimPlate Yeast & Mold - Color Indicator is validated by both MicroVal and AOAC-OM with a validated time to result of 72 hours, shorting the time required for a result by 48 hours compared to the reference method. The kit is sold in units of 100 tests with a maximum shelf life of 6 months with the approximate costs of 22 SEK per test (varies with the price of the euro). One component needs refrigeration. It is distributed in Scandinavia by Statens Serum Institut, Copenhagen, Denmark. According the MicroVal validation report it correctly enumerated all 32 target strains correctly and the tested 20 non-target strains did not show any reaction on the test. [MicroVal 2012] Figure 5 shows how a result from a test using SimPlate Yeast & Mold Color indicator.

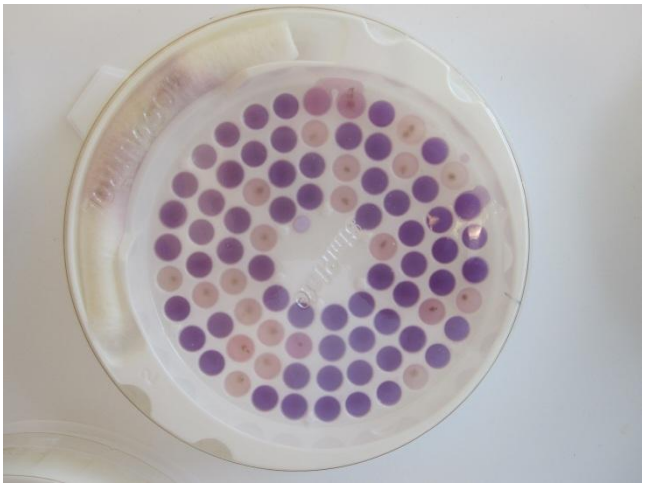

Figure 5: SimPlate Yeast & Mold Color indicator after performing a test. The wells that exhibit a color change from the original color (in this case blue) are counted and the number is converted to a MPN estimation.

4. Experimental setup

All three alternative methods were tested using products normally tested for *Salmonella* species and yeast and mold against the reference methods currently in use in the laboratory. Samples tested were selected together with the laboratory staff with regard to product category and to previous results in their routine testing. Fresh samples recovered directly from the production were also tested. Representative samples of 250g were collected from the products by the production staff and brought to the lab. All reference tests were performed according to the current methods section of this report (section 2.3.) and all alternative tests were performed according to the protocol supplied by the manufacturer (appendix A). All results were observed and counted manually.

All sterilizations were made by using the Getinge VS60 autoclave. Reusable laboratory supplies were washed by a Miele professional G7883 dishwasher. All samples were mixed using Stomacher bags with and without filters, depending on the particle size, in a Stomacher 400 circulator. In the case of products with large particles such as seeds the sample was mixed twice, once in the standard 30 second and then for another 2 minutes after allowed to rest for 30 minutes.

Necessary supplies for the alternative methods were supplied by respective distributor. For the reference method were all the XLD and YGC plates used pre-poured plates supplied by Oxoid AB, Malmö, Sweden. The peptone solutions and Rappaport-Vassiliadis broth were made from premixed dehydrated media supplied by Oxoid AB, Malmö, Sweden. All sample weighting were performed on an Inl smart dilutor. Greater dilutions were performed by mixing the diluted sample in Dilucups supplied by LabRobot Products AB, Stenungsund, Sweden.

Salmonella methods were tested in singlet and retested if a positive sample encountered. In the case of an alternative method testing positive both the alternative method and the reference method were retested. Isolated positive colonies were also restreaked on XLD plates and incubated for 24 hours and then read.

With the help from SIK – Institutet för Livsmedel och Bioteknik AB a small inclusivity and exclusivity test were performed on the *Salmonella* methods. Samples, broth and plates were delivered to SIK to be artificially inoculated by *Salmonella* species and other closely related Enterobacteriaceae. In addition a food sample (Polarn, batch 20935172) with a known high background flora was also brought to SIK to be artificially inoculated by different *Salmonella* species to test the interference from a high background flora. Both the artificially inoculated food samples from the production and the pure cultures were directly inoculated into the respective broths and then continued on with respective protocol.

Yeast and mold tests were performed in triplicates for all products and for both methods. To eliminate differences both methods used the same initially diluted sample which was further diluted as necessary. The references were tested in triplicates at the expected dilution with additional plates at the higher and, if applicable, lower dilutions. As a single SimPlate device test can detect a range from 1 to over 738 colonies it replaced two ordinary YGC plate dilutions. If unusual colonies were encountered these were isolated, stained and examined under a microscope to confirm fungal species. Two tailed paired t-tests were performed to compare the methods.

5. Results

The results are divided in sections for each microorganism and presented below.

5.1. *Salmonella* detection results

In total 30 products of several different product categories, see appendix B, were tested by both alternative methods and the reference method. One product was tested twice by mistake. All these samples were uninoculated.

For the vast majority of the products tested, 24 out of the 30, all methods showed a negative result for *Salmonella* indicating absence of *Salmonella* species in the products. One product, melon seeds, gave a positive reaction on both alternative methods but not on the reference method. Restreaking isolated positive colonies on XLD plates showed no positive reaction from any colony from either alternative method. When retesting the product both the reference method and IRIS Salmonella gave a negative reaction but Salmonella Precis gave yet again a positive reaction. Salmonella Precis tested positive on two additional products, millet grains and sesame seeds, where both the reference method and IRIS Salmonella showed negative reactions. Salmonella Precis did test positive yet again on the retest on the sesame seed but not on the millet grains. Restreaking showed no positive reactions on the XLD plates. IRIS Salmonella showed positive reactions on three additional products: two different batches of a nutrition soup (CA Potatis/purjolök) and poppy seeds. Both the retests and restreaking of isolated cultures on XLD plates gave a negative reaction however.

At SIK – Institutet för Livsmedel och Bioteknik AB seven strains of *Salmonella* (*Salmonella* Senftenberg CCUG 2880, *Salmonella* Montevideo CCUG 12648, *Salmonella* Dublin CCUG 24021, *Salmonella* Infantis ATCC 51741, *Salmonella* Enteritidis ATCC 25928, *Salmonella* Typhimurium CCUF 29478 and *Salmonella* Enteritidis) were used. Out of the seven strains of *Salmonella* it showed that while XLD cannot detect *Salmonella* species that are H_2S-negative such as *Salmonella* Senftenberg CCUG 2880 both alternative methods could accurately detect the serotype. However *Salmonella* Dublin was not detected by the Salmonella Precis alternative method, giving no signs of *Salmonella* while it produced very typical colonies on the XLD plate and gave a weak but clear indication on IRIS Salmonella agar. IRIS Salmonella managed to detect all tested serotypes of Salmonella. The results were the same for both the artificially contaminated sample and the pure culture. Pure cultures of Citrobacter, E.coli, Proteus, Klebsiella and Enterobacter were inoculated to the broths and spread to test for any cross reactions and no method showed any cross reaction for any microorganism.

From this data it was possible to calculate values for relative accuracy, relative sensitivity and relative specificity, see table 4 below.

Table 4: Performance indicators for the alternative methods.

Method	Positive agreement, PA	Negative agreement, NA	Negative deviation, ND	Positive deviation, PD	Number of tests, N	Relative accuracy, (PA+NA)/N (%)	Relative sensitivity, PA/(PA+ND) (%)	Relative specificity, NA/(NA+PD) (%)
Salmonella Precis	5	28	1	6	40	**82,5**	**83,3**	**82,4**
IRIS Salmonella	6	30	0	5	41	**87,8**	**100**	**85,7**

The inclusivity and exclusivity were as following (table 5):

Table 5: Inclusivity and exclusivity of the alternative methods.

Alternative method	Inclusivity	Exclusivity
Salmonella Precis	**6/7**	**5/5**
IRIS Salmonella	**7/7**	**5/5**

5.2 Yeast and mold enumeration results

A total of 23 products of several different categories were enumerated for molds and yeasts and the data is presented in appendix C. Some tests were unable to be read due to mold overgrowth of fast growing species (see figure 6 and figure 7) and these results are not considered. In four cases the mold overgrowth rendered either method unreadable and the products had to be excluded. The affected products were: Oriflame Choklad (1), Charkextra, Yellow linseed and Poppy seed (1). In three products only one replicate were affected and the following product's data are based on two replicated rather than three on one of the methods: Oriflame Choklad (3), Porridge and Sunflower seed. In one product, Poppy seed (2), two replicates were affected on both methods and thus this product's data is only based on one replicate on each method.

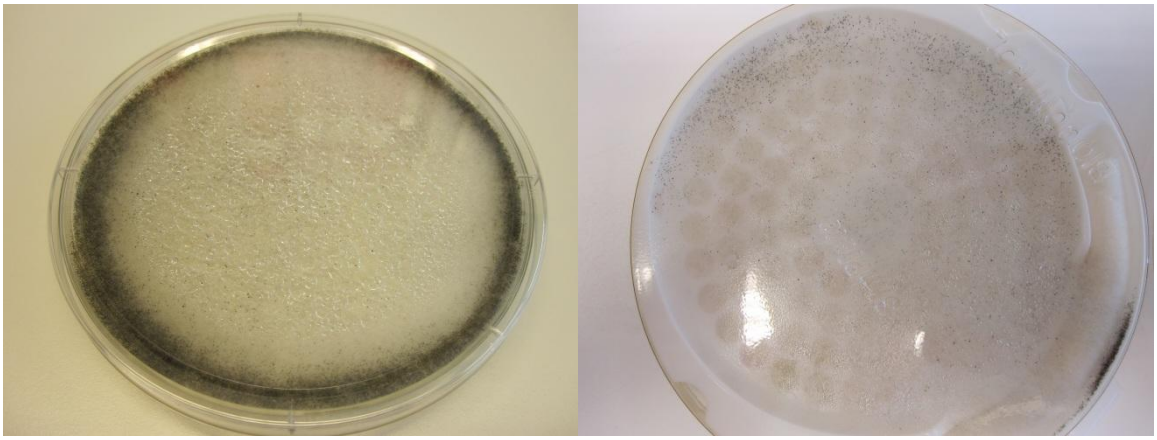

Figure 6 (left) and figure 7 (right): Examples of mold overgrowth which renders the result unreadable. Figure 6 feature the reference method and figure 7 feature the SimPlate device.

Below is a graphical representation of the data obtained divided into to two figures, figure 8 for lower counts (<1000) and figure 9 for higher counts (>1000).

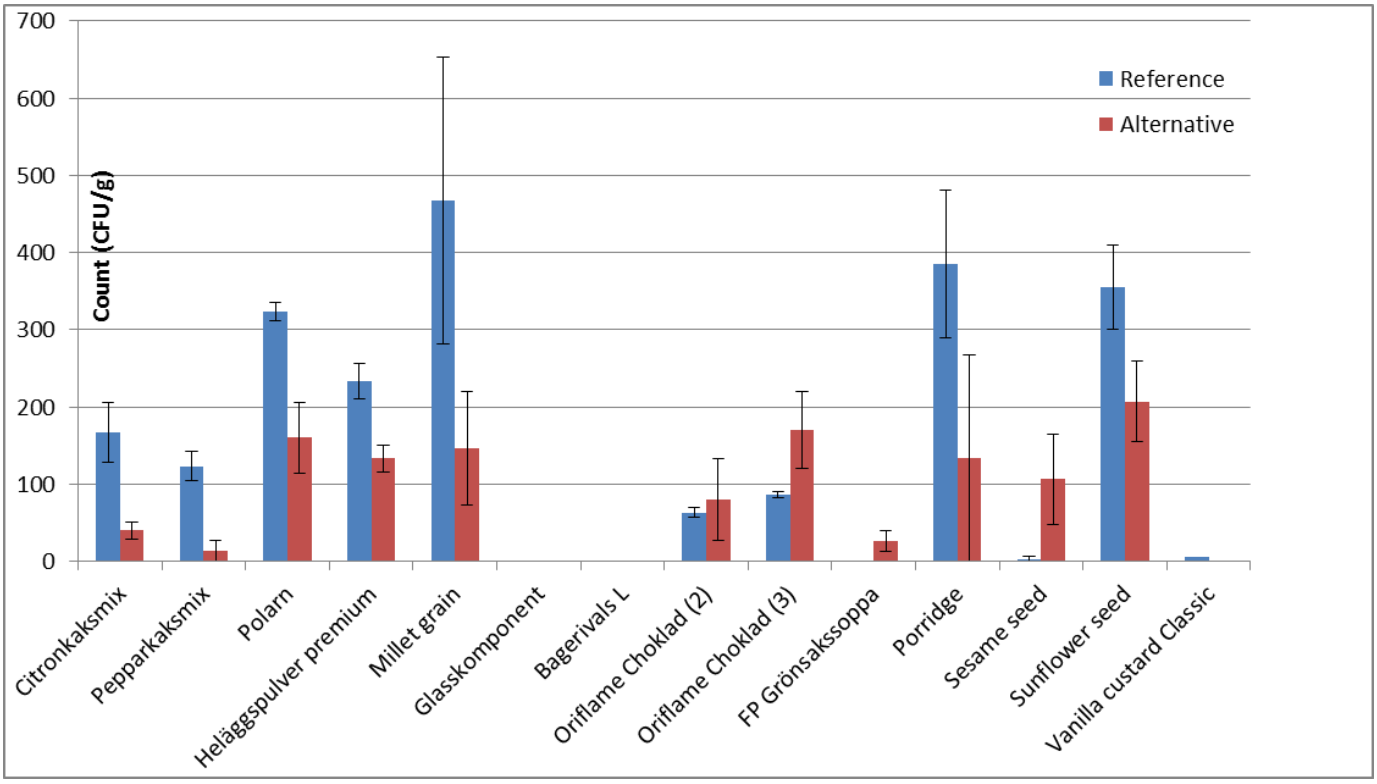

Figure 8: Graphical representation of the data with lower counts (<1000) obtained in the yeast and mold enumeration experiment. The error bars represent one standard error of the mean in both directions.

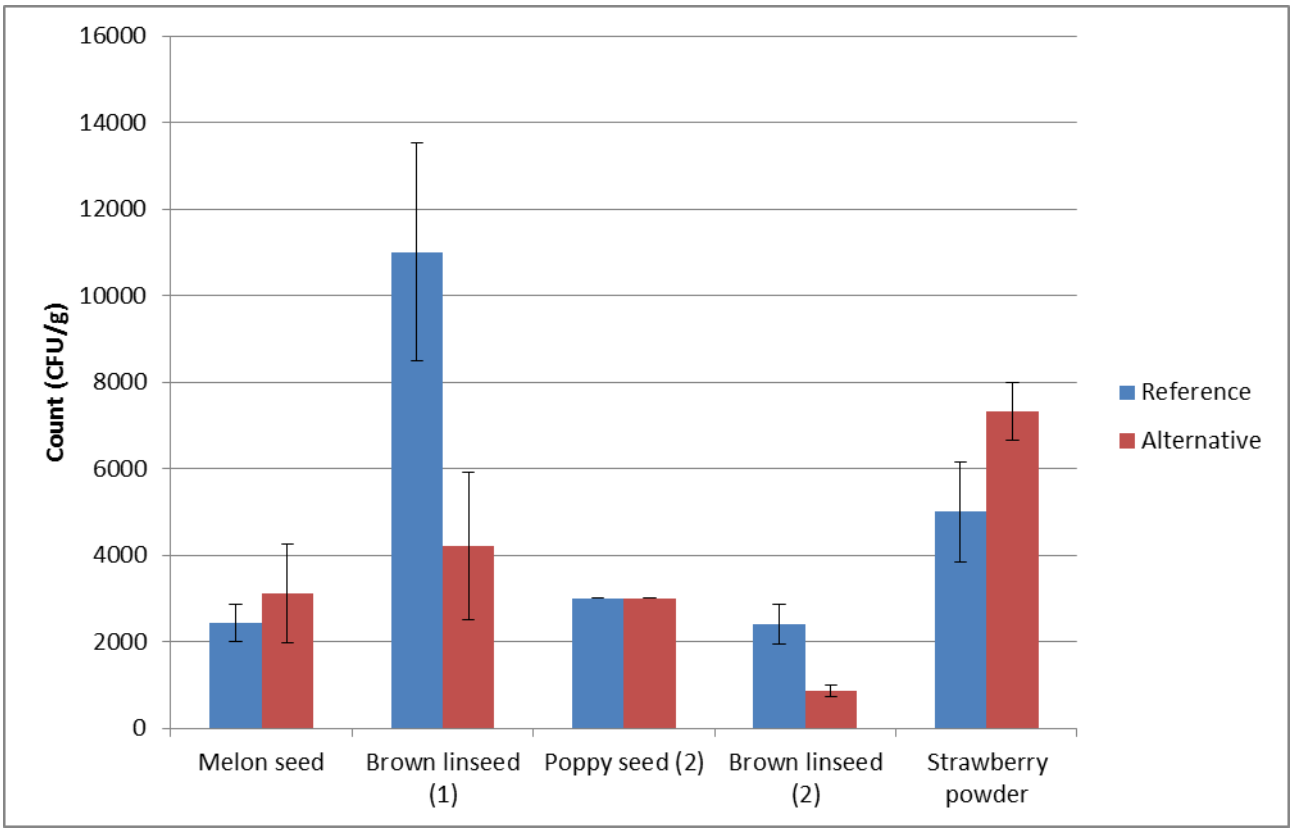

Figure 9: Graphical representation of the data with higher counts (>1000) obtained in the yeast and mold enumeration experiment. The error bars represent one standard error of the mean in both directions.

For nine products, Citronkaksmix, Pepparkaksmix, Polarn, Heläggspulver premium, Millet grain, Porridge, Sunflower seed, Brown linseed (1) and Brown linseed (2), the alternative method underestimated the enumeration compared to the reference method. For four products, Oriflame Choklad (3), FP Grönsakssoppa, Sesame seed and Strawberry powder, the alternative method overestimated the enumeration. Only in six cases, Glasskomponent, Bagerivals L, Oriflame Choklad (2), Vanilla custard Classic, Melon seed and Poppy seed (2), were there a reasonable similarity between the methods. In three of these cases, Glasskomponent, Bagerivals L and Vanilla custard Classic, were the fungal populations undetected or very low.

For one product, Heläggspulver premium, the difference was statistically significant at the 95% level of significance (p-value ≤ 0.05) using a two-tailed paired t-test. Three additional products were significantly different at the 90% level of significance (p-value ≤ 0.10); these were Citronkaksmix, Pepparkaksmix and Polarn.

6. Discussion

The amount of experiments is not as high as I wanted it to be since I did not have the time to test more products. This is mostly due to me underestimating the time it would take for the distributors to provide me with accurate information about their methods. This forced me to wait longer than expected before a decision could be made and I could start with the experiments. I tried to go directly to the manufacturer with my product related inquiries but they referred me back to the local distributors. The time requirement for the reference methods also limited when I could perform experiments as I did not have access to the laboratory on weekends or holidays due to the laboratory being closed. The reference method for *Salmonella* requires 4 consecutive days which only left Monday and Tuesdays for starting new experiments for weeks without holidays. For yeast and mold I wanted to use the same diluted sample for both tests to minimize the differences as much as possible which meant I had to start them at the same day and the only day which the 72 hour and 120 hour incubation could both be accommodated without either ending on the weekend was Friday. As the laboratory staff also needs access to the lab to perform their tasks that was also a limiting factor.

6.1. *Salmonella* detection methods

According to the results of the experiments IRIS Salmonella did seem to perform better than Salmonella Precis at almost all performance indicators used with higher scores in relative accuracy, relative sensitivity, relative specificity and inclusivity. In the exclusivity test both methods correctly did not detect any *Salmonella*. These findings coincide with the AFNOR validation report findings (table 3) that also found IRIS Salmonella to detect a broader range of *Salmonella* species than Salmonella Precis. Both methods did find a few false positives compared to results of the reference method. At the retests of the product that either method showed positive only Salmonella Precis showed the same positive result. This can be due to the presence of *Citrobacter diversus* or *Enterobacter Sakazahii* (*Cronobacter Sakazakii*) which showed cross reaction with Salmonella Precis in the AFNOR validation exclusivity test. [AFNOR 2012]

Looking at the practicality the IRIS Salmonella test again has an edge over Salmonella Precis due to it uses the tablets for application instead of relying on a freeze dried pellet and thus there is no need to prepare sterile water for this method. The coloration agent present in the tablet makes it easy to

distinguish which media that already have been supplemented however the downside is that the strong coloration makes it harder to observe if the tablet has been dissolved completely. It was also found that the tablets dissolved less readily than the freeze dried pellet of the Salmonella Precis method. IRIS Salmonella offers more flexibility in the protocol (Appendix A, A.1.) than Salmonella Precis (Appendix A, A.2.).

IRIS Salmonella were also the cheaper alternative especially when buying higher numbers. The price for IRIS Salmonella does not factor in eventual discounts if B. Engelhardt & Co AB becomes a recurring costumer which could lower the cost even more. However since the product is only to be used as a complement and the fact that B. Engelhardt & Co AB would still want to use the reference method as much as possible since it is considerably cheaper with a cost per test of around 8 SEK it is unlikely that buying in larger packages is viable due to the rather short shelf life of the products. The larger packages of plates will also take up additional space in the refrigerator. From these considerations and results I would recommend the use of IRIS Salmonella over Salmonella Precis.

Comparing IRIS Salmonella with the reference method there is the obvious advantage that the alternative method is 24 hours faster than the reference method. It also saves operator handling time. The initial dehydrated media preparation step is identical and it can be argued that the reference method requires less time than the alternative method due to the use of smaller volumes for the alternative method as the media is divided to smaller beakers of 225 ml prior to autoclavation. However the enrichment step is considerably faster for the alternative method as it is simply to add a tablet to the sample while for the reference method there is a need to prepare and autoclave Rappaport-Vassiliadis broth. It also saves time due to the transfer from pre-enrichment to enrichment does not occur in the alternative method. The downside is that the alternative method is only about 90% accurate due to false positives compared to the reference method; if false-positives occur it will need confirmation that will take up additional time. This especially a problem since the alternative method is to be used when a faster result is desirable. IRIS Salmonella (about 50 SEK/test) is also more expensive to use compared to the reference method (about 8 SEK/test). It is ultimately up to the quality division at B. Engelhardt & Co AB to take this into consideration and decide if the advantages weigh up the drawbacks.

6.2. SimPlate Yeast & Mold Color Indicator

The results from the yeast and mold experiments are varied. For nine products the alternative method underestimated the count compared to the reference method. For one of these products the difference is even statistically significant ($p<0.05$) and three products were close to be significantly different ($p<0.10$). This is a problem due to the quality test aims to check that the fungal population is below a certain limit depending on product. If the alternative method tends to underestimate it can never be certain if the product is in fact below the specified limit. If it instead would overestimate then it would be reasonable to approve products which are below the limit by the alternative method as it then very likely to be even more below the limit. However if the overestimation is greatly exaggerated it would falsely indicate that a product has an unacceptable fungal population when in fact it is acceptable. For the rest of the tests there were a small overestimation for four products, none of which were significant, and six products where the results are reasonable similar. Half of these reasonably similar results had little to none fungal population and one is only based on one replicate per method.

The underestimation could have been due to the strong coloration of certain products which could mask a color change in the wells, however no such trend could be seen as the method underestimated products with and without coloration agents. In the products with the strongest coloration, Oriflame Choklad (2) and Oriflame Choklad (3), the alternative method showed no underestimation.

Other studies on the SimPlate Yeast and Mold Color Indicator have also shown varied results. In an experiment on non-pasteurized orange juice SimPlate correlated well with the acidified PDA media as reference but did not show as strong correlation with DG-18 media as reference. [Ferrati, *et al.* 2005] Another more comprehensive study found that SimPlate Yeast and Mold Color Indicator tended to overestimate the fungal count in the products they tested and also found significant differences in the results. [Tournas, Rivera Calo and Memon 2011]

The greatest problem when dealing with the yeast and mold experiments was the overgrowth of fast growing mold species which rendered replicates unreadable. SimPlate Yeast and Mold Color Indicator is sold with certain supplements called “companion products” one of which is a supplement supposed to prevent the overgrowth of mold. However this information was only first encountered when reading the supplied manual after arrival of the actual product to the lab. Later on the manufacturer’s webpage was reworked and now contains a list of the “companion products” but still (as of 2012-05-15) does not specify their function. As the problem with the spreading mold was encountered in the testing the supplement against mold overgrowth was ordered. However the distributor did not have it in stock and it failed to arrive before the experiments had ended so the effect of this supplement was not tested. The content of the supplements are undisclosed and protected by the manufacturer as are the media. Tournas, Rivera Calo and Memon (2011) also reported problems with spreading molds when testing the SimPlate Yeast and Mold Color Indicator.

When looking at the practicality the alternative method does give a result in only 72 hours which is 48 hours faster than the reference method. However the handling time is longer for the alternative method than the reference method due to the media are delivered in pre-filled plastic tubes which only contain enough dehydrated media for one test making preparing media for larger batches impractical. As the plastic tubes cannot be autoclaved sterile water has to be prepared separately. The media also does not dissolve readily when mixing in a vortex and requires supplementation which adds to the handling time. However the SimPlate devices have a larger counting range per plate with one device replacing two YGC plates. The alternative method also does not require bent sterile glass rods for spreading as the reference method does.

As one device can replace two plates the cost increase is not as dramatic as for the *Salmonella* methods. The alternative method is approximately twice the price of the reference method, approximately 22 SEK/test against approximately 11 SEK/test, when using one device to replace two plates. However since the alternative method does use up more handling time the actual cost is greater.

Considering these results and the results from other studies it is not possible at this point to conclude that the alternative method and reference method gives reasonably similar results. Both in these experiments and in the experiments conducted by Tournas, Rivera Calo and Memon (2011) statistically significant differences were found.

7. Conclusions

According to the results in these experiments and that of the AFNOR validation IRIS Salmonella performs better than Salmonella Precis on the performance indicators, it offers more practically and is less expensive to use. From these data the use of IRIS Salmonella is recommended over Salmonella Precis. Comparing IRIS Salmonella to the reference method it can be concluded from the experiments that IRIS Salmonella offers 24 hours faster results while being easy to use and saves operator time. It also offers a good inclusivity and is AFNOR validated. On the downside however it only is about 90% accurate relative to the reference method and is considerably more expensive. It is up to the quality division at B. Engelhardt & Co AB to decide if this is acceptable or not.

The SimPlate Yeast and Mold Color Indicator gives a result 48 hours faster than the reference. However it was not possible to conclude that the alternative method gives reasonably similar results to that of the reference method. It tends to underestimate the count compared to the reference; some of these underestimations were also significantly different from the count of the reference method. It is laborious to use, takes up operator time and is more expensive. The method also suffers from problems with spreading mold but so does the reference method. The method could not be recommended for use.

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Appendix A: Protocols for the selected methods

A.1. IRIS Salmonella

A.1.1. Manufacturer's description

Studies performed with **IRIS Salmonella® Agar** have demonstrated its high level of specificity for *Salmonella* detection, including atypical species and serovars that may be the origin of confusing or conflicting results on other media. In this context, the detection of *Salmonella* Typhi & Paratyphi, lactose-positive *Salmonella* (*Salmonella* Senftenberg, as well as sub-species *S*. arizonae & *S*. diarizonae), and saccharose-positive strains are possible with the IRIS Salmonella system. The medium also allows for the detection of immobile serovars (*S*. Pullorum & *S*. Gallinarum) or monophasic strains that have lost mobility.

IRIS Salmonella® Agar permits the detection of strains only weakly esterase positive, or even negative on other esterase-based detection media (*Salmonella* Bongori, *Salmonella* Dublin and Atento, as well as various strains of the sub-species *S*. houtenae & *S*. diarizonae). A judicious blend of selective agents insures proper inhibition of Gram positive bacteria and certain Gram negatives.

A.1.2. Preparation of *Salmonella* Enrichment

- Add 20.0 g of dehydrated media (BK194) to 1 liter of distilled or demineralized water.
- Mix well, until complete dissolution.
- Divide into tubes or vials.
- Sterilize by autoclaving at 121°C for 15 minutes.

A.1.3. Instructions

- Introduce 25 g of the sample to be tested aseptically into 225 mL of the prepared *Salmonella* Enrichment broth.
- Mix well.
- Add an IRIS Salmonella tablet to the broth + sample mixture. The dissolution of the tablet takes place very rapidly and can be observed by the progressive coloration of the broth to green.

Note 1: The tablet can be added to a volume of *Salmonella* Enrichment ranging from 225.0 to 238.5 mL. This allows for the testing of between 25.0 and 26.5 g for product.

- Incubate **IRIS Salmonella® Enrichment** at 41.5 ± 1.0°C for 18 ± 2 hours.

Note 2: For reasons of organization within the testing laboratories, incubation can be extended to 24 hours. In the same context, broth cultures can be kept for 3 days at 2-8°C before being inoculated onto **IRIS Salmonella® Agar**.

- Inoculate 10 µL of the selective enrichment onto **IRIS Salmonella® Agar**.
- Incubate at 37.0 ± 1.0°C for 24 ± 3 hours.

Note 3: Again for reasons of laboratory organization, the agar plates can be kept for 3 days at 2-8°C before being read and prior to eventual confirmation via latex or other means.

- Confirm rose to magenta colonies as *Salmonella* spp. by appropriate biochemical and serological methods.

A.2. Salmonella Precis

A.2.1. Manufacturer's description

An Inhibigen compound is comprised of two components, combined together by a bond that can only be cleaved by a specific enzyme. When bound together, the inhibitor compound is not toxic and therefore can exist in a medium without harming micro-organisms. Once inside the cell, the bond will be cleaved if the target enzyme is present. When the bond is cleaved, the inhibitor molecule is released and disrupts cell wall synthesis, causing death of the organism. As cells die and lyse, free inhibitor is released but cannot be taken up by other cells, resulting in targeted inhibition. The Inhibigen in *Brilliance* Salmonella Agar targets *Escherichia coli*. Novobiocin and cefsulodin, supplied as a freeze-dried supplement (SR0194), are added to the medium to inhibit the growth of other competing flora such as *Proteus* spp. and *Pseudomonas* spp.

Differentiation of *Salmonella* from the other organisms that grow on *Brilliance* Salmonella Agar is achieved through the inclusion of two chromogens that also target specific enzymes: caprylate esterase and ß-glucosidase. Caprylate esterase is an enzyme present in all samonellae as well as some species of *Klebsiella*, *Enterobacter* and *Proteus*. Organisms possessing caprylate esterase cleave the chromogen to release an insoluble purple chromophore. As the cells grow, the chromophore builds up and produces a purple-coloured colony. Some Enterobacteriaceae, including *Klebsiella* and *Enterobacter*, but not *Salmonella*, possess ß-glucosidase. If these organisms grow, they will form blue or dark blue colonies, even if they are esterase positive, which make them easy to differentiate from purple *Salmonella* colonies.

A.2.2. Preparation of ONE Broth-Salmonella

225ml: Suspend 5.6g of ONE Broth-Salmonella Base in 225 ml of distilled water.

2.25 liters: Suspend 56.3g of ONE Broth-Salmonella base in 2.25 l of distilled water.

Sterilize by autoclaving at 121°C for 15 minutes. Cool to below 50°C.

Re-suspend the contents of 1 vial of ONE Broth-Salmonella Supplement (SR0242E) in 2 ml sterile distilled water and mix gently. Add the suspended supplement to 225 ml sterile medium and mix well.

Note: The vial contains novobiocin – avoid contact with the skin. Wear suitable gloves.

A2.3. Instructions

- Add 25g or 25ml of food sample to 225ml of ONE Broth-Salmonella.
  For solid samples, stomach for a minimum of 30 seconds to mix the sample.
- Incubate the broth at 42± 1°C for 18h ± 2h
- Gently agitate the bag then, using a 10µl microbiological loop, inoculate the broth onto a *Brilliance* Salmonella (OSCM II) plate using a diminishing sweep technique to produce single colonies.
- Incubate the plates at 37°C for 24h ± 2h
- Confirm purple colonies as *Salmonella* spp. by appropriate biochemical and serological methods.

A.3. Simplate Y&M-CI

A.3.1. Manufacturer's description

SimPlate for Yeast and Mold Color Indicator (Y&M-CI) method is used for the detection and quantification of yeast and mold in foods. It is based on Binary Detection Technology (BDT) which equates the presence of yeasts and mold to the presence of a color change in the medium. The medium is inoculated with a prepared sample, dispensed into a SimPlate device and incubated for a minimum of 56 h. The medium changes color in the presence of yeast and/or mold. The yeast and mold count is determined by counting the wells with changed color and referring to the SimPlate Conversion Table.

A.3.2. Instructions

- Weigh 10g of sample into 90 ml of sterile 0.1% peptone water diluent. This is a 1:10 dilution. Masticate to homogenize. If necessary, prepare 10-fold serial dilutions appropriate for the anticipated population of the sample.
- **1:10 dilution**: Resuspend powdered medium with 9.0 ml of sterile deionized water containing 0.09 ml of Supplement A (1 ml/100ml). Add 1.0 ml of sample and mix well. DO NOT count this reconstitution as a dilution.
- **1:100 dilution**: Resuspend powdered medium with 9.9 ml of sterile deionized water containing 0.099 ml of Supplement A (1ml/100ml). Add 0.1 ml of sample and mix well
- The final volume of sample/medium mixture in the container should be 10 ml ± 0.2 ml.
- Remove the lid from the SimPlate device and pour the sample/medium mixture onto the center of the plate. Immediately replace the lid.
- Gently swirl to distribute the sample/medium mixture into all the wells. The plate may be held with both hands and tilted slightly to help distribute the liquid into the wells.
- Pour off excess medium by holding the lid against the plate on either side of the sponge cavity. Tip the plate toward you to allow liquid to drain into the sponge. Examine wells to ensure that they are completely filled. Observe the background color of the wells. Background is defined as the color of the sample/medium mixture inside the wells.
- DO NOT invert the SimPlate device. Incubate at room temperature (22-25°C) for 72 h in the dark.
- After incubation, observe color change of the liquid in the wells. Disregard particulate matter if present. Count the number of wells showing a color change from the background color. The most common color changes produced by fungi are red, white, peach and orange.
- To determine the population, perform the following calculations:
    - Count the number of positive wells on the plate.
    - Use the SimPlate Conversion Table to determine the total number of fungi per plate.
- To calculate the number of fungi per sample, multiply the count by the appropriate dilution factor.

A.3.3. SimPlate Normal Counting Range (NCR) Conversion table

Number of positive wells = populations per plate

The population reflects the number of microorganisms per plate. To determine the number of microorganisms per gram (mL) food product, multiply the count by the appropriate dilution factor.

1	=	2	29	=	70	57	=	190
2	=	4	30	=	74	58	=	196
3	=	6	31	=	76	59	=	202
4	=	8	32	=	80	60	=	208
5	=	10	33	=	84	61	=	216
6	=	12	34	=	86	62	=	224
7	=	14	35	=	90	63	=	232
8	=	16	36	=	94	64	=	240
9	=	18	37	=	96	65	=	248
10	=	22	38	=	100	66	=	256
11	=	24	39	=	104	67	=	266
12	=	26	40	=	108	68	=	276
13	=	28	41	=	112	69	=	288
14	=	30	42	=	116	70	=	298
15	=	32	43	=	120	71	=	312
16	=	36	44	=	124	72	=	324
17	=	38	45	=	128	73	=	338
18	=	40	46	=	132	74	=	354
19	=	42	47	=	136	75	=	370
20	=	46	48	=	142	76	=	392
21	=	48	49	=	146	77	=	414
22	=	50	50	=	150	78	=	440
23	=	54	51	=	156	79	=	470
24	=	56	52	=	160	80	=	508
25	=	58	53	=	166	81	=	556
26	=	62	54	=	172	82	=	624
27	=	64	55	=	178	83	=	738
28	=	68	56	=	184	84	=	>738

If there are no positive wells and the sponge is positive, populations = 1. If there are no positive wells and the sponge is negative populations is <1.

Appendix B: Salmonella data

Batchno.	Product name	Category	First test			Retest		
			Reference	Salmonella Precis	IRIS Salmonella	Reference	Salmonella Precis	IRIS Salmonella
85791810	Äpplebitar eko	Apple bits	-	-	-	N/A	N/A	N/A
20897280	Citronkaksmix	Bakery	-	-	-	N/A	N/A	N/A
20936280	Wasa premix	Bakery	-	-	-	N/A	N/A	N/A
20897280	Citronkaksmix	Bakery	-	-	-	N/A	N/A	N/A
13353898	Pepparkaksmix	Bakery	-	-	-	N/A	N/A	N/A
21285188	Polarn	Bread improver	-	-	-	N/A	N/A	N/A
21285187	Polarn	Bread improver	-	-	-	N/A	N/A	N/A
20394335	Charkextra	Charcuterie	-	-	-	N/A	N/A	N/A
EX3205	Heläggspulver premium	Egg powder	-	-	-	N/A	N/A	N/A
R120024	Millet grain	Grain	-	Detected	-	-	-	N/A
20824384	Glasskomponent	Ice cream	-	-	-	N/A	N/A	N/A
21154414	Bagerivals L	Milk derived	-	-	-	N/A	N/A	N/A
21283243	CA Potatis/purjolök	Nutrition soup	-	-	Detected	-	N/A	-
21283244	CA Potatis/purjolök	Nutrition soup	-	-	Detected	-	N/A	-
21283245	FP Vegetable	Nutrition soup	-	-	-	N/A	N/A	N/A
20933165	CA LCD/HP CREAMY CHOCOLATE	Nutriton drink	-	-	-	N/A	N/A	N/A
20933166	CA LCD/HP CREAMY CHOCOLATE	Nutriton drink	-	-	-	N/A	N/A	N/A
20933167	CA CHOKLAD	Nutriton drink	-	-	-	N/A	N/A	N/A
20833144	FAIRING RECOVER R2 CHOKO TWIST	Nutriton drink	-	-	-	N/A	N/A	N/A
20753128	FAIRING COMPLETE CHOCOLATE	Nutriton drink	-	-	-	N/A	N/A	N/A
21243239	INDEVEX NGC BASE MIX/SHAKE MIX	Nutriton drink	-	-	-	N/A	N/A	N/A
21243238	INDEVEX NGC BASE MIX/SHAKE MIX	Nutriton drink	-	-	-	N/A	N/A	N/A
R120022	Poppy seed	Seed	-	-	Detected	-	N/A	-
R120023	Yellow linseed	Seed	-	-	-	N/A	N/A	N/A
R120025	Sesame seed	Seed	-	Detected	-	-	Detected	N/A
R120026	Sunflower seed	Seed	-	-	-	N/A	N/A	N/A
R120027	Melon seed	Seed	-	Detected	Detected	-	Detected	-
R120036	Poppy seed	Seed	-	-	-	N/A	N/A	N/A
20893161	Vanilla custard Classic	Vanilla custard	-	-	-	N/A	N/A	N/A
20893161	Vanilla custard Classic	Vanilla custard	-	-	-	N/A	N/A	N/A

Appendix C: Yeast and mold data

Batchno.	Name	Category	Alt. 1	Alt. 2	Alt. 3	Alt. Mean	Ref. 1	Ref. 2	Ref. 3	Ref. Mean	p-value
20897280	Citronkaksmix	Bakery	20	60	40	**40**	200	210	90	**166,7**	0,084
13353898	Pepparkaksmix	Bakery	40	<10	<10	**13,3**	100	110	160	**123,3**	0,062
20665138	Polarn	Bread improver	80	160	240	**160**	300	340	330	**323,3**	0,051
20394335	Charkextra	Charcuterie	160	140	160	**153,3**	N/A	N/A	N/A	**N/A**	N/A
EX3205	Heläggspulver premium	Egg powder	140	100	160	**133,3**	210	210	280	**233,3**	0,023
R120024	Millet grain	Grain	<100	220	220	**146,7**	100	600	700	**466,7**	0,107
20824384	Glasskomponent	Ice cream	<10	<10	<10	**<10**	<10	<10	<10	**<10**	N/A
21154414	Bagerivals L	Milk derived	<10	<10	<10	**<10**	<10	<10	<10	**<10**	N/A
21098143	Oriflame Choklad (1)	Nutrition drink	N/A	N/A	N/A	**N/A**	500	400	300	**400**	N/A
12447195	Oriflame Choklad (2)	Nutrition drink	60	<10	180	**80**	50	70	70	**63,3**	0,442
12447196	Oriflame Choklad (3)	Nutrition drink	120	N/A	220	**113,3**	80	90	90	**86,7**	0,310
21153221	FP Grönsakssoppa	Nutrition soup	<10	40	40	**26,7**	<10	<10	<10	**0**	0,184
21133215	Porridge	Porridge	<100	<100	400	**133,3**	480	290	N/A	**385**	0,154
R120022	Poppy seed (1)	Seed	N/A	N/A	N/A	**N/A**	N/A	N/A	N/A	**N/A**	N/A
R120023	Yellow linseed	Seed	<100	N/A	N/A	**N/A**	500	N/A	N/A	**500**	N/A
R120025	Sesame seed	Seed	200	<10	120	**106,7**	<10	10	<10	**3,3**	0,233
R120026	Sunflower seed	Seed	200	300	120	**206,7**	300	N/A	410	**236,7**	0,289
R120027	Melon seed	Seed	5400	2080	1840	**3106,7**	3300	2100	1900	**2433,3**	0,445
R120028	Brown linseed (1)	Seed	2600	2400	7600	**4200**	16000	9000	8000	**11000**	0,212
R120029	Poppy seed (2)	Seed	3000	N/A	N/A	**3000**	3000	N/A	N/A	**3000**	N/A
R120030	Brown linseed (2)	Seed	600	960	1040	**866,7**	3200	2400	1600	**2400**	0,122
R1200276	Jordgubbspulver	Strawberry powder	9600	6200	6200	**7333,3**	5000	3000	7000	**5000**	0,286
20893161	Vanilla custard Classic	Vanilla custard	<10	<10	<10	**<10**	<10	10	10	**6,7**	0,184