Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences

Implementation of HACCP plan for blue-mould tofu with focus on microbial hazards

Genomförande av en HACCP-plan för blåmögel tofu med fokus på mikrobiella faror

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Department of Molecular Sciences
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Abstract

There is an increased request for vegan alternatives among consumers. This may depend on several factors, in many cases related to health and ethics. Food safety is very important to achieve in food production and there are several food safety management systems that can be applied to ensure that the food can be considered safe. Therefore, the first task in this study is to conduct a microbial analysis on a new vegan product, blue-mould tofu, to identify pathogens. The second task is to implement a HACCP (Hazard Analysis and Critical Control Points) plan for the product, with focus on microbial hazards. The purpose is to evaluate which procedures should be in place to ensure that the product is safe to consume. The product consists of soy and faba beans, and has similar sensory properties as blue-mould cheese. The production has been done at SLU (Swedish University of Agricultural Science) in Uppsala Sweden, on a research level meanwhile this study was conducted. A literature review was performed to find out which microbial pathogens could be an issue in the product. A laboratory analysis was performed on cheeses from three different batches to identify pathogens in the product. The information from the literature review and the laboratory analysis was used to implement a HACCP plan with focus on microbial hazards. No pathogens were identified in the product except low counts of *B. cereus*. The results indicated that the product has a high hygienic status. Some batch variation was observed since growth of unidentified bacteria was more extensive in the first batch compared to the two others. It transpired that this happened in large part due to improved cleaning and personal hygiene during production. Boiling of bean milk is also a crucial step to ensure food safety, together with pH monitoring and control of raw ingredients (soy and faba beans). These three steps were identified as CCPs (Critical Control Points).

Keywords: HACCP, food safety, foodborne pathogens, tofu, cheese

Sammanfattning

Det finns en ökad efterfrågan av veganska livsmedel bland konsumenter. Detta kan bero på flera orsaker bland annat av hälso- eller etiska skäl. Livsmedelssäkerhet är mycket viktigt att uppnå i livsmedelsproduktionen och det finns flera livsmedelssäkerhetssystem att tillämpa med syfte att producera säkra livsmedel. Första uppgiften i denna studie är att genomföra en mikrobiologisk analys på en ny vegansk produkt, blå-mögel tofu, för att identifiera patogena mikroorganismer. Andra uppgiften är att utveckla en HACCP-plan (Hazard Analysis and Critical Control Points) för produkten, med fokus på mikrobiella risker. Syftet är att utvärdera om produkten är säker att konsumera. Produkten består av soja och åkerbönor, och har liknande egenskaper som blåmögelost. Produktionen sker under detta arbetes gång på en forskarnivå vid SLU (Sveriges Lantbruksuniversitet) i Uppsala. En litteraturstudie genomfördes för att ta reda på vilka patogena mikroorganismer som kan orsaka problem i produkten. En laboratorieanalys utfördes på ostar från tre olika produktionsomgångar med syfte att identifiera patogena mikroorganismer i produkten. Informationen från litteraturgenomgången och laborationsanalysen användes för att upprätta en HACCP-plan med fokus på mikrobiella risker. Inga patogena mikroorganismer identifierades i produkten förutom ett lågt antal *B. cereus*. Resultaten indikerade att produkten har en hög hygienisk status. Vissa variationer mellan produktionsomgångarna observerades då tillväxten av oidentifierade bakterier var mer omfattande i det första partiet jämfört med de andra. Det visade sig att detta berodde till stor del på grund av förbättrad rengöring och personlig hygien under produktionen. Åtgärder som visade sig vara viktiga för att säkerställa livsmedelssäkerheten var värmebehandling av bönmjölken, pH mätning samt mottagningskontroll av bönor. Dessa tre steg identifieras som CCPs (kritiska kontroll punkter).

Nyckelord: HACCP, livsmedelssäkerhet, livsmedelsburna patogener, tofu, ost

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Abbreviations

CCP	Critical Control Point
Cfu	Colony forming unit
FDA	US Food and Drug Administration
GHP	Good Hygiene Practice
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis and Critical Control Points
ISO	International Organization for Standardization
PRP	Prerequisite Program
JTI	Swedish Institute of Agricultural and Environmental Engineering
LAB	Lactic Acid Bacteria
SLV	Swedish National Food Agency
Spp	Species

1 Introduction

1.1 Background

The demand for vegan foods has lately increased. The reason that individuals chose to follow a vegan diet may depend on many factors such as health and ethics. Reasons associated with allergies or the fact that there is a greater supply of vegan foods on the market can also explain the trend. The health aspect can be seen as one of the main reasons why people may chose a vegan diet (Radnitz, Beezhold & DiMatteo, 2015). Vegan foods contain high levels of dietary fibers, several vitamins such as vitamin C, E and folic acid and minerals such as potassium and magnesium. The fat content is also beneficial due to its low grade of saturation (Winston, 2009). A good vegan alternative that possesses high nutritional value is legumes. Legumes are considered to be an over all advantageous food mainly with respect to their high protein and fiber content, which also makes them a good substitute for food of animal origin. They are also a good source of, besides nutrients mentioned above, non-digestible carbohydrates. Another advantage is their versatility since many products can be made from legumes (Rebello, Greenway & Finley, 2014).

Soy is due to its high nutritional value and low cost, a popular oil seed crop and protein source, cultivated worldwide (Hartman et al., 2016). Soy is LDL-cholesterol free and considered to decrease the risk for heart diseases (Serrazanetti et al., 2013; Chen et al., 2014). Soy has good functional properties and can be processed to many products, of which one of the most popular products is tofu, a traditional Asian product (Rossi et al., 2016). The method for producing tofu has been the same for hundreds of years. Main steps in the production are soaking soybeans in water, grinding, and separating of liquid (milk) and solid (okara) phase. The milk is then boiled, filtered and inoculated with coagulant such as magnesium or calcium sulfate, or magnesium or calcium chloride. The coagulation then begins and results in a curd, which is pressed into preferred shape (Ananchaipatttana et al., 2012). Like soybeans, faba beans contain high amounts of proteins and other beneficial nutrients. Faba beans have also the advantage to be able to grow in different climates (Crepon et al., 2009). Faba beans are today mainly cultivated for animal feed.

To meet the higher demand for vegan and locally produced food, a new product is developed, a blue-mould tofu made from soy and faba beans. The initiative was started by Vinnova, which is a Swedish innovation agency with purpose to strengthen the innovation power in Sweden by funding research and innovation-projects. Vinnova's new venture is about producing protein-rich foods that in the future can be an alternative to conventional animal-based production (Vinnova, 2016a). One of those products is the blue-mould tofu that has similar sensory properties to a blue-mould cheese. Ingredients used are soybeans and Swedish faba beans. This product would have lower negative impact on the environment than milk based products like cheese, and other positive outcomes include an increased demand for faba beans, which are now only produced for animal feed in Sweden. The product is at this point produced in a laboratory scale by SLU and JTI in the department of food science at SLU (Vinnova, 2016b).

Food safety is very important to consider in all settings which in some way handle foods that are destined for consumption. That can be all types of companies, from primary production to restaurants. There are several food safety management systems, which facilitate the work to produce safe food and decrease the risk of food borne diseases. It is required for every food producer to maintain a HACCP plan, with the aim to ensure that the produced food is safe to consume (Teixeira & Sampaio, 2013). Tofu is a product susceptible to microbial growth (Rossi et al., 2016), therefore there is a need for a well conceived food safety plan. The blue-mould tofu will be studied with respect to hazards, mainly microbiological hazards to ensure that the product will be safe to consume. The product will be analyzed for microbial pathogens and a HACCP plan with focus on microbiological hazards will be implemented, mainly applicable on small scale/research level.

1.2 Aim and limitations

This work will consist of a laboratory analysis of pathogens in the blue-mould tofu. Since this is a new developed product and every food production needs a food safety management system, a HACCP plan will also be implemented. The literature review covers information about reported microbial pathogens in soybeans, faba beans, tofu and cheese, to get a broad perspective on which microorganisms could pose a problem in the blue-mould tofu. The blue-mould tofu is currently produced at a laboratory scale/research level, therefore the main focus will be on microbiological hazards. It may be difficult to determine all chemical and physical hazards that may occur in a future commercial production environment.

2 Literature review

2.1 Food safety management

Food safety is essential to achieve in order to prevent food borne diseases and improve public health. The increased demand for foods with long shelf life and high safety status, together with the increased number of reported causes of foodborne diseases calls for well-implemented food safety management systems (Sandrou & Arvanitoyannis, 2000). Several food safety management systems are available with the aim to produce safe food in all types of food companies. However this is a challenge, especially for small industries due to several factors, such as lack of education. ISO 22000:2005 is a standard published in 2005 by ISO. This standard is applicable in all type of companies and organizations in the food chain and involves both requirements in ISO 9001 and the practices described in HACCP (Teixeira & Sampaio, 2013).

2.1.1 ISO 22000

An increasing global trade increases the requirements for food safety and quality management. ISO 22000 is an international collection of standards with focus on food safety, of which ISO 22000:2005 covers the overall guidelines for food safety management (ISO, 2015). The guidelines are applicable throughout the whole food production chain. The standards are designed in a common language, which facilitates both understanding and communication between different operators in the food chain. The methodology includes the following key factors: interactive communication, system management, basic conditions and HACCP-principles (Bergström & Hellqvist, 2006).

2.1.2 HACCP

HACCP (Hazard analysis and critical control points) is an internationally accepted and used system to ensure food safety by identifying and preventing hazards in food production. The method covers the entire food processing chain and aims to be used by companies that produce and/or in some way handle food that is destined for consumption (Soman & Raman, 2016). The basic principles are identification, evaluation and control of food safety hazards. HACCP can be associated with food quality, however it mainly covers food safety and is therefore not a quality system according to ISO 9001, which states that a quality system has to manage all quality aspects (Bergström & Hellqvist, 2004). According to article 5 in regulation (EC) 852/2004, is it required to implement and maintain a HACCP system by food operators in all kind of food companies (EC, 2004).

Methodology of HACCP is based on seven principles (EC, 2005):

1) Hazard analysis – identifying all possible hazards
2) Identifying critical control points (CCPs)

3) Establish critical limits at every CCP
4) Establish monitoring procedures
5) Establish corrective actions
6) Establish verification procedures
7) Establish documentation and records to demonstrate effectivity of measures outlined in 1-6.

Hazard analysis is done to identify possible hazards in the production. A hazard is a "*source of danger; defined as a biological, chemical or physical property with the potential to cause an adverse health effect*" (Adams & Moss 2014, 426). To achieve a proper hazard analysis the product and production process need to be carefully described. All ingredients, production steps and equipment must be taken into account, and then eventually the possible hazards that can occur at every step are listed (EC, 2005). The consideration of the occurrence of hazards can be based on previous studies, epidemiological data and experience (FDA, 2014). Preventive measures are included for every hazard, this step can include measures such as cleaning routines or temperature controls. Every hazard should then be evaluated whether it is a CCP or not; to facilitate this step, a decision tree can be used at every production step (EC, 2005). CCP is a step in the food processing chain where control is needed with the aim to eliminate, prevent or reduce the hazard to a accepted level (Lamboni, Sanaa & Tenenhaus-Aziza, 2014). Identification of CCPs is a fundamental step in the development of a HACCP plan.
When CCPs are identified, critical limits are established for each point. A critical limit is a value that separates acceptable (safe) from unacceptable (not safe). Monitoring procedures are necessary to control the process and detect deviations. This can be done by observations and measures such as microbiological sampling, temperature control and cleaning controls. If a critical limit is not met, corrective actions must be taken, which is an action plan used when the limits are exceeded. Verification is done to verify if the system works properly (EC, 2005; Holmberg & Wallin 2010, 24-34). There are different alternatives for verification procedures; in many cases, measures could include a review of the HACCP plan or sampling and testing of the CCPs carried out. Verification should proceed routinely to ensure that that the system is under control. Documentation routines should be implemented to facilitate detection of deviations and to ensure that the system works properly (FDA, 2014).

Prerequisite programs
Prerequisite programs (PRPs) are basic guidelines designed to ensure that the produced foods are safe, healthy and of high quality. PRPs are described in various regulations and guidelines, and include food safety and quality measures. PRPs should be developed prior a HACCP plan to ensure that all regulations and requirements for the product are met. The requirements are designed in a general and in an open-ended way, which gives the manufacturer flexibility to apply them in a way that fits the production. The program involves quality and safety assurance systems along the food chain, with aim to maintain control over production and to meet consumers'

requirements for safe food with high quality. PRPs include Good Manufacturing Practices (GMP) and Good Hygiene Practices (GHP). Such practices include personal hygiene, water, cleaning and sanitation, pest control, education, temperatures, supplier control, design of facilities and waste disposal (FDA, 2014).

2.2 Applicable regulations for novel foods

Novel foods must be approved in two steps before they can be released on the market. First step is the approval of new food, which includes safety testing. The next step involves notification of a food product that is similar to an already approved product. A list of already existing foods is available in the European Commission's novel food catalogue. The safety testing is done in the nation where the application is submitted. In Sweden, the food producer sends the application to the Swedish National Food Agency. The agency makes a preliminary approval and forwards the application to the European Commission (SLV, 2016).

New food that has not been consumed to a significant degree before 15 May 1997 must be risk assessed and approved by the European Commission before it can be placed on the EU market. The aim is to ensure that the foods are safe to consume. This is regulated by the EC regulation 258/1997 – concerning novel foods and novel food ingredients. The regulation includes new foods and ingredients that have not been used for human consumption. It may concern ingredients that have a changed or new molecular structure, ingredients consisting of microorganisms, fungi and algae, plant based ingredients and ingredients that have changed properties by a new production method. The regulation also includes evaluation of the food and marking (EC, 1997). In January 2018 this regulation will be replaced by EC regulation 2015/2283 on novel foods.

The blue-mould tofu production steps and ingredients are similar to traditional tofu production. The mould used for ripening is the same as for blue cheese production. The products do not include any new ingredients that have not been used before, which makes the blue-mould tofu a product development rather than a novel food. Swedish National Food Agency has during the course of this work agreed that the blue-mould tofu is not a novel food, but is classified as a product development. However the regulations applicable for novel foods may be necessary to apply on similar products if they are produced with new ingredients or methods.

2.3 Potential food safety hazards from microbes associated with soya and faba beans

2.3.1 In field and during storage

Soybean

Aspergillus, Penicillium and *Fusarium* are common genera of fungi responsible for food spoilage. These can produce several mycotoxins toxic for humans, and therefore they can, besides spoilage,

pose a health risk in food (Hymery et al., 2014). Soybeans are frequently infected by fungi, both during cultivation and post-harvest. Factors affecting growth and toxin production are humidity, dew and temperatures (temperatures above 25 °C promote growth and toxin production).

Fusarium and *Alternaria* are considered to be the most common fungi isolated from soybeans, according to several studies. These moulds can infect the soybean seeds. *Alternaria* spp. are often present in soil and damaged plant tissues, it is also a common post-harvest pathogen (Barros et al., 2011). *Alternaria alternata* is the most common species found in soybeans. *Alternaria* spp. produces mycotoxins such as alternariol monomethyl ether (AME) and alternariol (AOH). Both toxins have shown potential mutagenic, genotoxic and carcinogenic effects (Oviedo et al., 2011), however, there are currently no regulations for mycotoxins produced by *Alternata* spp. in food (EFSA, 2011). The growth of AME and AOH depends to a great degree on water activity and temperature in the environment. A study showed that the greatest production of AME and AOH occurs at a water activity around 0.98 and at temperatures around 25-30 °C, these values depend though on each other and on other factors such as the strain. Since mycotoxins can be classified as microbiological hazards in soybeans, the knowledge about affecting parameters such as water activity and temperature should be taking into account when designing storage facilities and the processing environment (Oviedo et al., 2011).

Fusarium spp. produces a broad spectrum of mycotoxins of the class trichothecenes: nivalenol, T-2 toxin, HT-2 toxin (Creppy, 2002) and deoxynivalenol (DON), which is the most distributed in food. DON causes symptoms such as vomiting, diarrhoea and nausea (Adams & Moss, 294). The toxin is very heat stable and is not destroyed at 100 °C, although processing at higher temperatures (above 120 °C) leads to degradation, which also depends on the pH (Kabak, 2009). It has been shown that DON mainly contaminates the soybean in the field, with reported levels at 0.9 μg/g– 1.6 μg/g (Barros et al., 2011). Zearalenone and fumonisins are other toxins produced by *Fusarium* spp. Zearalenone can be produced in soybeans both in the field and during storage. The toxin has shown potential carcinogenic properties in human. Fumonisins (FBs) have also shown some toxic effects in humans such as carcinogenic effects and harmful impacts on the fetus (Creppy, 2002). According to Gacia et al. (2014), fumonisins have been detected in dry soybeans at 138 to 1495 μg/kg and 178 to 552 μg/kg for fumonisin B_1 and B_2, respectively. There are no set limits for FBs in soybeans. Besides, there is still a lack of studies regarding mycotoxin contamination in soybeans compared to other cereals (Garcia et al. 2016).

Aspergillus spp. has also been detected in soybeans during the production process (Barros et al., 2011). The main mycotoxin produced by *Aspergillus* spp. is aflatoxin, which is mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins can be produced in the bean during storage due to improper storage conditions. They are considered to be one of the most carcinogenic natural compounds, which can cause liver cancer (Adams & Moss 2014, 282-288). There are about twenty aflatoxins, of which B_1, B_2, G_1, and G_2 are the most common in foods

(Lee, Heer & Lee, 2015). *A. flavus* produces only B aflatoxins, while *A. parasiticus* produce both B and G aflatoxins (Creppy, 2002). Aflatoxins are heat-stable, however, in a study the levels decreased remarkably when soybeans were heated at 100 and 150 °C for 90 min, in fact by 41.9% and 81.2% respectively (Lee, Heer & Lee, 2015).
Actually most of the mycotoxins are heat stable within the temperature range applied for food processing (80-121 °C). Some factors that affect the level of degradation during heat processing are pH, water content, temperature and concentration of mycotoxins (Kabak, 2009).

According to a study made by Garrido et al. (2013), the most common isolated fungi from soybeans are *Cladosporium, Chaetomium, Sclerotinia, Alternaria, Aspergillus, Penicillium, Phomopsis* and *Fusarium.* Studies above indicate that soybeans are susceptible for infection by various fungi both pre and post-harvest.

Salmonella have also been isolated from soybeans used for animal feed production. *Salmonella* has the ability to grow in various environments and on different materials, therefore must a food safety plan for products consisting of soybeans be carefully evaluated when it is developed (Wierup & Kristoffersen, 2014).

Faba bean
Faba beans are susceptible to diseases caused by airborne fungi, soil-borne pathogens related to foot and root rot complexes, viruses, parasitic weeds and nematodes. The biggest reported problems in faba beans are associated with cultivation. Diseases that can affect the faba bean plant are among others; rust, ascochyta blight, chocolate spot, cercospora leaf spot, downy mildew (Sillero et al., 2010).

There is a lack of studies available about processed faba bean foods, since faba beans are mostly cultivated for animal feed. Therefore, the assumption in this study is that the potential pathogenic microorganisms in processed faba beans are similar to those in processed soybeans described above.

2.3.2 In tofu and similar products
Tofu is a good growth medium for microorganisms due to its high water- and protein content. The shelf life is relatively short, actually only a few days. Lactic acid bacteria, *Enterobacteriaceae* and *Pseudomonas* are the major spoiling bacteria in tofu (Rossi et al., 2016). Because of the bacteria-friendly properties of tofu there is also a risk for pathogens to grow. Contamination of tofu with pathogenic bacteria such as *L. monocytogenes* has been reported. In a study regarding microorganisms in packed and unpacked tofu in Thailand, certain bacteria were detected. The major gram-negative bacteria included coliforms, *Pseudomonas* spp. and *E. coli*, while the major gram-positive bacteria included *Enterococcus* spp., lactic acid bacteria, *Bacillus cereus* and *Staphylococcus* spp. Unpacked tofu contained higher amounts of coliform bacteria such as *Salmonella* spp., *Enterococcus* spp. and *E. coli,* than packed tofu. These bacteria were

considered to originate from the environment, humans and animals (Ananchaipatttana et al., 2012).

B. cereus has been detected in both packed and unpacked tofu; since the bacteria produce enterotoxins, it is considered a health risk. *B. cereus* form spores, which can be present in soybean seeds, and furthermore survive the production steps of tofu (Ananchaipatttana et al., 2012). Prestamo et al. (2000) reported that *B. cereus* was present in cheese even after high-pressure treatment. *B. cereus* is spore forming, gram-positive and rod shaped bacteria. It can grow in temperatures between 7 and 55 °C, spores can survive much higher temperatures. Infection shows symptoms as abdominal pain, diarrhoea, vomiting and rectal tenesmus. The bacteria are spread in the environment and can be found in soil, water and vegetation (Adams & Moss 2014, 185-190). Therefore it is a risk for growth in soybeans used in tofu production but also in raw milk used for cheese production.

Other bacteria such as *Yersinia* spp., *Pseudomonas* spp. and *C. sakazakii* have also been detected in tofu. These bacteria can be inactivated by heat treatment of the product. As mentioned earlier tofu is a great medium for microorganisms, which means that the processing and handling should be performed carefully to reduce the risk of spoilage. Proper storage temperatures and good hygiene practice during production and storage are crucial factors to produce tofu with high quality and food safety status (Ananchaipatttana et al., 2012).

Lactic acid bacteria (LAB) has been added to tofu with the aim to prolong the shelf life. This has shown good results, with decreased contamination and longer shelf life of tofu. The mechanism behind this is that LAB produce antimicrobial molecules, for instance acetic acid, 2-nonen-1-ol, limonene and benzyl alcohol. Another property of LAB is production of lactic acid and inhibition of the peroxidation of unsaturated fatty acids, which also prolongs the shelf life (Chen et al., 2014; Serrazanetti et al., 2013).

2.4 Potential food safety hazards from microbes associated with cheese production

2.4.1 Microbial hazards and spoilers in cheese

Cheese is considered as an overall safe food due to the fact that milk is often pasteurized before cheese production and thus free from non spore-forming pathogens. However that depends on cheese and production-type since the intrinsic and extrinsic factors can vary (Prencipe et al., 2010). Cheeses contain a high numbers of different microorganisms such as bacteria, moulds and yeast. Most of them contribute to the specific aroma, taste, consistency and appearance of cheese (Banjara, Suhr & Hallen-Adams, 2015). However it is important to keep in mind that some of these organisms can spoil or contaminate the cheese and cause negative consequences.

Bacteria

Escherichia coli, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes* are potential pathogenic microorganisms in cheese, and their incidence has been reported in numerous studies. They can be found in raw milk and in some cases survive during cheese production. Even though the contamination often originates from the raw milk, there are various sources where the contamination of cheese can occur. Such sources can be: starter culture, equipment, storage facilities, package material, humans and air (Prencipe et al., 2010; Kousta et al., 2010; Choi et al., 2016).

E. coli belongs to the *Enterobacteriaceae* family and is a gram-negative, non spore-forming and rod-shaped bacterium. It ferments sugar and grows at temperatures from 7-10 °C up to 50 °C, with an optimum at 37 °C. pH optimum is around 7, but the growth can continue down to pH 4.4 (Adams & Moss 2014, 216-224). Enterovirulent *E. coli* are divided in four categories; *Enterotoxigenic* E. coli (*ETEC), Enteroinvasive* E. coli (*EIEC), Enterohaemorrhagic* E. coli (*EHEC)* and *Enteropathogenic* E.coli (*EPEC).* These bacteria cause similar symptoms in the gastrointestinal tract, of which the most characteristic is, severe diarrhoea. Particulary *EHEC*, sometimes referred to as *VTEC*- verotoxinproducing *E. coli*, can cause life-threatening infections such as haemorrhagic colitis, haemolytic uraemic syndrome and thrombotic thrombocytopaenim purpura.

Salmonella belongs, as *E. coli* to the *Enterobacteriaceae* family. It is a gram-negative, rod-shaped and non spore-forming bacteria. Growth appears at temperatures from 5 to 47 °C with an optimum at 37 °C. They are heat sensitive, which means that their presence in cheese is decreased by pasteurization and other heat treatments, such as boiling. Optimal pH is 7, but growth can occur down to around pH 4 (Adams & Moss 2014, 226-228). *Salmonella* cause different syndromes categorized after enteritis and systemic disease. Incubation time for enteritis is normally between 6 and 48h. Infection appears as flu-like symptoms, vomiting, diarrhoea and abdominal pain. *Salmonella* spp. and *E. coli* are often introduced in raw milk (Prencipe et al., 2010), where the major source of contamination is the farm environment, for example feaces (Kousta et al., 2010).

S. aureus is a coccal, gram-positive and non spore-forming bacteria. It grows at temperatures between 7 and 48 °C with an optimum at 37 °C, optimum pH for growth is 6-7 but can also occur at values of 4 and 9.8-10.0 (Adams & Moss 2014, 252-257). *S. aureus* occurs in many places in the environment, mainly on the human and animal skin. Food poisoning has a short incubation time, around 2-4 h, and the symptoms include vomiting, nausea, stomach cramps, retching, prostration and in some cases diarrhoea. Poisoning is caused by ingestion of food containing one or more enterotoxins. The biggest contamination source of *S. aureus* in raw milk is infected udders, which often lead to an excretion to the milk (Kousta et al., 2010). The growth of *S. aureus* can be controlled in cheese making by different measures such as proper temperatures and pH (Carrascosa et al., 2016). The bacteria are tolerant to salt and reduced water activity (Adams &

Moss 2014, 254), which has to be kept in mind when producing cheese, a product that often possesses these properties. *Staphylococcus* spp. can indicate the hygienic levels in raw milk, during handling and storage, and can thus be used as a hygienic parameter for cheese production (Prencipe et al., 2010).

L. monocytogenes is a non spore-forming, gram positive and rod-shaped bacteria that grows at temperatures between 0-42 °C, with an optimum between 30 and 35 °C (Adams & Moss 2014, 226-228). Results in Prencipe et al. 2010, showed that blue-mould and soft cheese varieties are prone to *L. monocytogenes* contamination. *L. monocytogenes* is mainly an environmental contaminant. It can grow and survive over long periods at refrigeration temperatures, which make it a concern (Kousta et al., 2010). In most cases the growth stops at pH 5.5 and the bacteria has shown a tolerance to high salt concentrations. The bacteria exist in different environments such as water and soil (Adams & Moss 2014, 226-228).

L. monocytogenes can cause the food-borne infection listeriosis. Pregnant women's, newborns, elderly and people with weak immune system are particularly susceptible to listeriosis. For those groups the infection can in worst case be fatal. The symptoms can vary but include in most cases flu-like illness with fever, headache and gastrointestinal problems. These bacteria's can form biofilms on equipment and processing environment. Therefore cleaning, disinfectant and good hygiene practice are particularly important to avoid contamination (Kousta et al., 2010). According to Lamboni, Sanaa & Tenenhaus-Aziza (2014), CCPs for *L. monocytogenes* in soft cheese production are environment of ripening facilities, cheese making phase, transportation and conservation. Factors that are important to control are the environment quality, level of contamination, and temperature during transport. Suitable temperature during transportation and conservation is 4.3-5.5 °C and during ripening 11.4-13.5 °C. According to Silva, Alves & Almeida (2003), *Listeria* was isolated from raw milk, environment, and floors. Identified CCPs in that study were the raw milk, heat treatment, coagulation and storage.

Yersinia enterocolitica is another potential pathogen in cheese, particularly in blue cheese (Zadernowska, Chajecka-Wierzchowska & Ogryzek, 2015). *Y. enterocolitica* is spread in the environment, and can for instance be found in water, soil and the intestinal tract of many animals (Adams & Moss, 264). The bacteria is non spore-forming and gram negative. It is psychotropic and can grow at temperatures between 1 and 40 °C, with at optimum at around 29 °C. Optimal pH is around 4-8. The source of contamination of cheese may be the raw milk, which can in turn be contaminated during improper milk processing or re-infection (Kowalik & Robacz, 2015). If the cheese becomes contaminated, the bacteria are able to grow during storage under a long period, and cause food poisoning (Zadernowska, Chajecka-Wierzchowska & Ogryzek, 2015). The infection has an incubation time between 1-11 days and the most common symptoms are diarrhoea, abdominal pain and mild fever (Adams & Moss 2014, 262-167).

As mentioned in the section about soy, *B. cereus* is a wide spread bacteria forming spores that endure heat-treatment, therefore it also is a concern for the dairy industry. Source of contamination is in most cases the raw milk, but also processing environment. The cheese making process includes favorable conditions, such as heating and slow cooling, for spore activation and subsequent growth of vegetative cells (Spanu et al., 2016).

Moulds

Moulds can enter the cheese in different ways such as by the starter culture, air, equipment or workers. Many of them as mentioned above are necessary to the cheese characteristics, although some of them can produce mycotoxins and can be a risk for disease. There are a wide variety of moulds that can spoil cheese, and also varieties that can produce mycotoxins. Mould variety depends to a great extent on the cheese variety. *Penicillium roqueforti* and *Penicillium camemberti* are two known moulds widely used in mould cheese production. Other *Penicillium* spp. are often found in cheeses as spoilers and some varieties are mycotoxin producers (Banjara, Suhr & Hallen-Adams, 2015), e.g. *P. nordicum* which is salt-tolerant and produces ochratoxin A (Schmidt-Heydt et al. 2012). *Aspergillus* spp. are also mycotoxin-producing moulds, of which *A. flavus* can be present in cheese, as well as in foods of plant origin (Banjara, Suhr & Hallen-Adams, 2015).

The main concerns with moulds are their ability to produce mycotoxins. Cheeses can be contaminated with mycotoxins from different sources such as raw milk from an animal that has ingested contaminated feed, from moulds added as a starter culture, or just by a spontaneous contamination. The health effects of mycotoxins depend on several factors such as the dose, organ infected and host properties as age and sex. Mycotoxicosis can in some cases be fatal (Sengun, Yaman & Gonul, 2008). However, there no are reported cases of fatalities from mycotoxins from cheese (Hymery et al., 2014). Studies have shown that certain mycotoxins are carcinogenic, though more studies are needed to determine the health risks of mycotoxins in dairy products. The most common mycotoxins in cheese are aflatoxin, penitrem A, roquefortine C, citrinin and sterigmatocystin (Sengun, Yaman & Gonul, 2008). Contamination with aflatoxins often originates from contaminated feed, the toxin is then excreted to the milk which is used for cheese production. Other mycotoxins can be formed by mould growth on the cheese, where moulds used as starter cultures start to produce mycotoxins. Roquefortine is produced by some strains of *P. roqueforti*, a typical species used in mould cheeses (Benkerroum, 2016). Citrinin can be produced by *P. camemberti*, but also in many cases by *Aspergillus* spp., which means that citrinin can enter the cheese in different ways. Sterigmatocystin is produced by some species of *Aspergillus*, where the contamination source may be the environment (Hymery et al., 2014). Penitrem A is produced by *Penicillium* spp. mainly *P. crustosum*, which has been found in different foods, feed and food waste. Intoxication with penitrem A has mainly been detected in animals (Tiwary, Puscher & Poppenga, 2009).

The mycotoxins mentioned above can be present in the final product since they can survive the production environment. The fundamental measure to avoid mycotoxins in cheese is inhibiting mould growth in all stages of production and storage (Sengun, Yaman & Gonul, 2008). Mould do not survive pasteurization temperatures (72 °C, 15 s), although ascospores produced by some moulds can survive heat at 90°C for 5 min. Reducing mould contamination can be done by different measures such as proper hygiene during production, both when it comes to handling the cheese and cleaning of equipment and storage facilities. Another important factor is to ensure that the milking cows ingest non-contaminated feed of high quality to prevent contamination already at that point. It has been shown that certain microorganisms such as lactic acid bacteria strains are able to bind to certain mycotoxins and by that inhibit their uptake in the intestine (Benkerroum, 2016). A low storage temperature of the final product, 5-7 °C, inhibits mould growth and mycotoxin production (Sengun, Yaman & Gonul, 2008), which is also important to take into account in this study. Kure, Skaar & Brendehaug (2004), found that the main contaminants in semi-hard cheese production were *Penicillium commune, P. palitans, P. roqueforti* and *P. solitum, P. brevicompactum* and *G. candidum* were isolated from the indoor environment, and were estimated to be the main moulds in the factories. In that study the primary source of contamination was the air, which also was an important control point during production (Kure, Skaar & Brendehaug, 2004). To avoid mould contamination from the air, the production rooms should have functioning ventilation systems. The environment should as mentioned earlier be kept clean. Clean clothes of the workers and good hygiene practice are also important factors. These measures can be applied in all types of food production to minimize the risk for mould contamination.

Yeasts

As for the moulds, yeasts can contribute to the cheese characteristics, mainly in varieties such as Camembert and blue-veined cheeses. Many types of yeast have the ability to inhibit unwanted microorganisms and support the starter culture by production of proteolytic and lipolytic enzymes. They have also an important role since they enhance the fermentation of lactose and utilization of lactic acid (Welthagen & Viljoen, 1998). *Debaryomyces hansenii* and *Galactomyces candidus* (a yeast-like mould, and teleomorph synonym of *G. candidum* mentioned above) are among other species two common yeasts in cheese. The environment in cheese is favorable for *D. hansenii* due to its tolerance of low pH and high salt concentrations. *G. candidus* is often used as a starter culture in surface mould ripened cheeses and in most cases is considered safe (Banjara, Suhr & Hallen-Adams, 2015). *Candida* spp. can also be present in cheeses. Some of them are of interest when it comes to human health, because of their ability to cause blood stream infection, although the highly pathogenic species do not appear in cheese to any significant degree (Jacques & Casaregola, 2008). Thus, the probability for yeasts to cause food borne diseases is small. However, certain yeasts can grow to a great degree in cheese and cause spoilage, which appears as changes in texture and flavors, developing of off-flavors, gas production and discoloration (Jacques & Casaregola, 2008). Sources of contamination have been shown not to be the raw

ingredients but mainly the processing environment such as equipment, walls, floor and hands (Welthagen & Viljoen, 1998).

2.5 Summary of potential microbially-derived hazards in blue-mould tofu

Based on the literature survey above, a summary of potential microbial hazards that should be taken in consideration during blue-mould tofu production is presented. Listed microorganisms have in previous studies been isolated from cheese, and/or soybeans and products made from soybeans. Both tofu and cheese seems to be relatively susceptible to contamination, in many cases with the same microbial pathogens.

Bacteria	***Moulds***
Salmonella	*Penicillium*
E. coli	*Aspergillus*
S. aureus	*Fusarium*
B. cereus	*Alternaria*
L. monocytogenes	

2.6 Microbiological criteria

The European Commission has established several regulations regarding food safety. Regulation (EC) No.2073/2005 on microbial criteria for foodstuffs, covers microbiological criteria for ready to eat foods including dairy products. The aim of the regulation is to protect a high level of human health by reducing risk for foodborne diseases, to verify HACCP measures and provide uniform criteria for all EU countries. The microbiological criteria have to be met by every food producer and followed up by regular samplings, analysis and corrective measures (EC, 2005). Sweden has not developed national microbial criteria since it was considered unnecessary, instead the EC regulation is followed. Established microbiological limits relevant for the blue-mould tofu are listen in Table 1. The regulations established by the European Commission do not include microbial criteria specific for tofu. However, the Australian and New Zealand Food Authorities have established limits for tofu in the Standard 1.6.1 of microbiological limits for food, which are included in table 1 (ANZFA, 2001). These values do not have to be followed in this case but can act as a guideline for the blue-mould tofu.

The following stated variables are seen in Table 1. n: Number of samples to be taken per food part, c: Number of samples where the result can exceed a certain limit (m) , m: Lower limit that can be exceeded in a number of samples (c), M: Upper limit that can not be exceeded.

Table 1. *Microbiological criteria relevant for blue-mould tofu*

Product/type of food	**Microorganism**	**Sampling plan (# of samples)**		**Limits (cfu/g)**	
		n	**c**	**m**	**M**
Cheeses made from milk that has undergone a lower heat treatment than pasteurisation and ripened cheeses made from milk or whey that has undergone pasteurisation or a stronger heat treatment	Coagulase positive staphylococci	5	2	10^4	10^5
Cheeses made from milk or whey that has undergone heat treatment	*E. coli*	5	2	10^2	10^3
Ready to eat foods	*Listeria monocytogenes*	5	0	10^2	
Tofu (not ultra heated)	*Bacillus cereus*	5	2	10^2	10^3
	E.coli	5	0	0	
	Coagulase positive staphylococci	5	2	10^2	10^3

(EC, 2005; ANZFA, 2001)

3 Materials and methods

3.1 Literature review and empirical study

Besides the laboratory identification of microorganisms in the blue-mould tofu, this work includes a literature review and an empirical study. Information for the literature review was retrieved from databases including Web of Science, PubMed and Scopus. Homepages of agencies and organizations such as SLV, FDA and Vinnova were also used. The empirical study was done by observations and collecting information about the blue-mould tofu. Information about the production process and product characteristics was collected by observation of the production process and from personal communication at the department of food science and department of microbiology at SLU.

3.2 Experimental

According to the literature review several microorganisms were considered to be a potential risk in the blue-mould tofu (Section 2.5). Bacteria tested in this lab were *S. aureus, E. coli, Salmonella, B. cereus* and *L. monocytogenes* (during the last trial). The methods for bacterial isolation were performed according to manuals in Nordic committee on food analysis; No. 71, 5th ed., No. 66, 3rd. ed., No. 144, 2nd. ed., No. 67, 4. ed. and No. 136, 5th ed. Since the product is inoculated with *Penicillium roqueforti,* bean milk and bean seeds were tested for moulds and yeasts instead of the final product. Identifications were performed at a genus level, the genera *Penicillium, Aspergillus* and *Fusarium* were looked for. The blue-mould tofu was produced in several batches in the lab. Cheeses from three different occasions were tested for bacteria. Twelve cheeses were tested in the first lab trial, four cheeses in the second and four cheeses cheese in the third and last trial. Bean milk was sampled from the last trial. See Appendix 2 for recipes for the different cheeses. The sample preparation was performed by the same method for all three trials. The reading and confirmation steps differed between the trials due to different bacterial growth.

Media preparation

Types of media used are presented in Table 2. Peptone water, buffered peptone water, Rappaport enrichment for *Salmonella* and Fraser broth (in half and full strength) was prepared in different amounts. Mannitol Salt Agar was used in the first trial for identification of *S. aureus* due to lack of material available. In the second and third trial Baird-Parker agar was used. See Appendix 1 for recipes.

Table 2. *Type of media and incubation conditions for respective microorganism*

Microorganism	**Media**	**Incubation**	
		Time	**Temp(°C)**
Salmonella	Xylose Lysin Desoxycholate (XLD)	24 h	37
	Brilliant green agar (BGA)	24 h	37
B. cereus	*Bacillus cereus* selective agar (BCSA)	24 h	30
S. aureus	Baird-Parker agar (BP)	48 h	37
E. coli	Violet red-bile-glucose agar (VRBG)	22-26 h	37
L. monocytogenes	PALCAM agar base	24 h	37
	ALOA agar	24 h	37
For re-streaking	Brain heart agar (BHA)	24 h	37
	Nutrient agar (NA)	24 h	37
	Blood agar	24 h	37
Aspergillus, Penicilluim & Fusarium	Malt extract agar	3-7 days	25

3.2.1 Bacteria

Sample preparation

25 g of each cheese was weighed and put in a stomacher bag. 225 ml peptone water was added and the bag was run in a stomacher machine for 120 s, with medium force. The samples were serially diluted to 10^{-1} to 10^{-3}, by mixing 1 ml sample in a tube with 9 ml peptone water.

Salmonella – qualitative detection

Pre-enrichment

10 g of each cheese was weighed and each placed in a sterile E-flask. 90 ml buffered peptone water was added to each flask. The samples were incubated at 37 °C for 24 h with slow stirring.

Enrichment

Tubes with Rappaport enrichment were pre-warmed in a water bath to 42 °C. 0.1 ml of pre-enrichment samples was transferred to each tube with enrichment. The tubes were incubated in a water bath at 42 °C for 24 h.

Salmonella inoculation

One loop of material from the enrichment broth was inoculated on XLD and BGA agar plates. The material was spread over the whole surface of the plate. The plates were incubated in inverted position at 37 °C for 24 h.

B. cereus, S. aureus and E. coli – quantitative detection

Inoculation

0.1 ml of each dilution was surface inoculated onto BP (MSA in the first trial) and BCSA. The plates were incubated in inverted position at 37 °C for 24 h. VRBG agar was melted and tempered to 45 °C in a water bath, 15 ml of agar was poured into plates and 1 ml of each cheese sample was inoculated. The inoculum was gently mixed and allowed to solidify. A thin layer of VRBG was poured on top of the solidified agar. After solidification of the last layer, the plates were incubated in inverted position at 37 °C for 24 h. All plates was checked after overnight incubation and re-incubated for another 24 h.

Reading and confirmation

Trial 1.

Samples from colonies on MSA, VRBG and BCAB plates were studied and observed under the microscope. One colony from each VRBG plate; 3 (all), 5 (10^{-2} and 10^{-3}), 2 (10^{-3}) and 1 (10^{-1}) were re-streaked on NA plates. One colony was re-streaked from BCSA plate 6 (10^{-3}) on a NA plate. Colonies were re-streaked from MSA plates 5 (10^{-1} and 10^{-2}) onto NA plates. All NA plates were incubated at 37 °C for 24 h. After incubation, samples from colonies on NA plates were studied under a microscope. As confirmation, gram test and oxidase test were performed on colonies from NA plates, which originated from VRBG and MSA plates. Gram test was done by adding few drops of 3 % KOH on a glass slide, where one loop of bacteria was added. If agglutination occurred the test was considered negative. Oxidase test was performed by placing a loop of bacteria on a test stick. The result was shown by a colour change on the test stick. If the colour appeared blue/red the result was positive and if the colour appeared yellow the result was negative.

Trial 2.

One suspected *B. cereus* colony from BCSA plate 9 (10^{-1}) was re-streaked on blood agar and incubated at 37 °C for 24 h. The following day, the morphology of the bacteria was confirmed by microscopy. A new re-streak was done on a NA plate. The plate was left in room temperature for 4 days. GLISA Duopath® Cereus Entorotoxins test was performed after 4 days, which is a rapid test for qualitative detection of *Bacillus cereus* enterotoxins. About 2 colonies were placed in 1 ml previously prepared BHI broth (1 % glucose). The sample was shaken and incubated for 4 hours in 37 °C. 150 microliter of sample was transferred to the testing device. The result was read after 30 minutes.

Trial 3.

In the last trial all the incubated plates were read after 24 h. Small dark and big slimy colonies from VRBG were examined microscopically, as well as some grey/brown colonies from BP. BactiStaph agglutination test was performed on colonies from BP, which is a selective confirmation test for *S. aureus*. 1 drop of BactiStaph Latex Reagent was placed on the slide.

Around 3 colonies were placed next to the reagent and gently blended with a supplied stirring stick. The slide was rotated in circular motion and the result could be observed within 60 seconds.

L. monocytogenes - qualitative detection

Primary enrichment

25 g of each cheese was placed in each stomacher bag. 225 ml Half-Fraser broth was added and the bag was run in the stomacher machine for 30 seconds. The suspensions were incubated at 30 °C for 24 h.

Secondary enrichment

0.1 ml of primary enrichment was transferred to each tube with 10 ml Fraser broth (full strength). These were incubated at 37 °C for 48 h.

Inoculation

One loop of secondary enrichment was inoculated on PALCAM and ALOA agar plates. The material was spread over the whole surface of the plate. The plates were incubated in inverted position at 37 °C for 24 h.

Reading

Colonies was examined on PALCAM and ALOA plates. Green colonies from ALOA were re-streaked on blood agar, incubated at 37 °C for 24 h.

3.2.2 Moulds

Sample preparation and inoculation

Bean milk

40 ml bean milk was collected from each cheese:

1. Clean milk (uncooked)
2. Clean milk (cooked)
3. Milk with 40% rape seed oil (uncooked)
4. Milk with rape seed and coconut oil (uncooked but warm)

The milk was serially diluted to 10^{-1} to 10^{-4}. 0.1 ml of sample was spread on MEA and incubated in upright position for 3-7 days at 25 °C.

Testing for endogenous infection in soy and faba beans

All equipment such as tweezers, two 250 ml Duran flasks, sieve and filter papers were autoclaved. 800 ml of 0.4 % NaOCl was prepared for sterilization of beans. Approximately 20 g of faba and soybeans were placed in each Duran flask. 0.4% NaOCl was added to a total volume of 200 ml. The flasks were left to stand for 2 minutes, with occasional mixing. The solution was discarded through a sieve and beans were allowed to dry. Filter papers were used to gently remove excess liquid. 9-10 beans were placed on each MEA plate using a tweezer (four plates for each bean

type). The plates were placed in plastic bags and incubated in upright position for 5-7 days at 25 °C.

Reading and confirmation

After 3 days, the plates with bean milk were checked for yeast growth. The susceptible colonies were counted and purified on MEA plates. MEA plates were divided into five sections and the colonies were re-streaked, small colonies on one plate and larger on the other. After 7 days, the plates with bean milk were checked for moulds. Some colonies from the re-streak from yeast-like colonies were observed in the microscope.

4 Empirical study

4.1 Product description

Table 3. *Product description*

Ingredients	Swedish soybeans and faba beans**,** commercial yogurt (thermophilic) starter culture**,** glucose monohydrate**,** commercial starter culture of *P. roqueforti***,** commercial microbially-derived protease**,** fat (rape seed oil and coconut oil) and salt.
Use and consumers	Ready to eat without need of heating before consumption. Intended for general public. Can be seen as primarily applicable to vegans and vegetarians, those allergic to dairy products, and consumers who want to decrease consumption of food of animal origin**.**
Package	Not designed yet. Most likely will be packed in the same way as similar products.
Labeling	Designed to ensure food safety.
Shelf-life	Not estimated at this point of development of the product.
Process-equipment/facilities	Mixer, saucepan, cutting knife, filter cloth, cup, bucket, water bath, cheese forms, incubator

Description of ingredients and the procedure

Glucose monohydrate is added to promote growth of lactic acid bacteria. The commercial thermophilic yogurt starter culture is prepared in bean milk to permit inoculation of pilot scale volumes used in this blue-mould tofu production. Lactic acid bacteria is added for fermentation. Lactic acid is produced during fermentation which contributes to decrease of the pH in the product and the taste; LAB is often used in yoghurt production. Low pH inhibits microbial growth, mainly bacteria, but is not as effective in inhibiting mould growth. The commercial microbially-derived protease is a microbial coagulant replacing common rennet, which contains the proteolytic and

milk-clotting enzyme aspartic protease. The mould starter culture is added for ripening and consists of the mould *Penicillium roqueforti*, which is common in blue-mould cheese production. Salt is added for taste and inhibiting growth of microorganisms. Fat captures flavors and contributes to the texture of the product. Two different sorts of fats of plant origin were tested with aim to see which is more favorable for the taste of the product.

In the mixing step (figure 1), 4,5 l water and beans are thoroughly ground and mixed using an Electrolux mixer. The bean milk is boiled with aim to destroy trypsin inhibitors in beans, and also to eliminate microorganisms. The yogurt starter culture, mould starter culture and microbial rennet are all added to the milk after it has cooled to an appropriate temperature favouring growth and survival of the starter cultures, but not too warm for the microbial rennet to make the coagulum too hard at higher temperatures. Piercing the cheese allows air to enter the cheese, which promotes growth of the mould culture. Humidity in the incubator is set to 70-80 %.

Figure 1. Blue-mould tofu

4.2 Flow chart

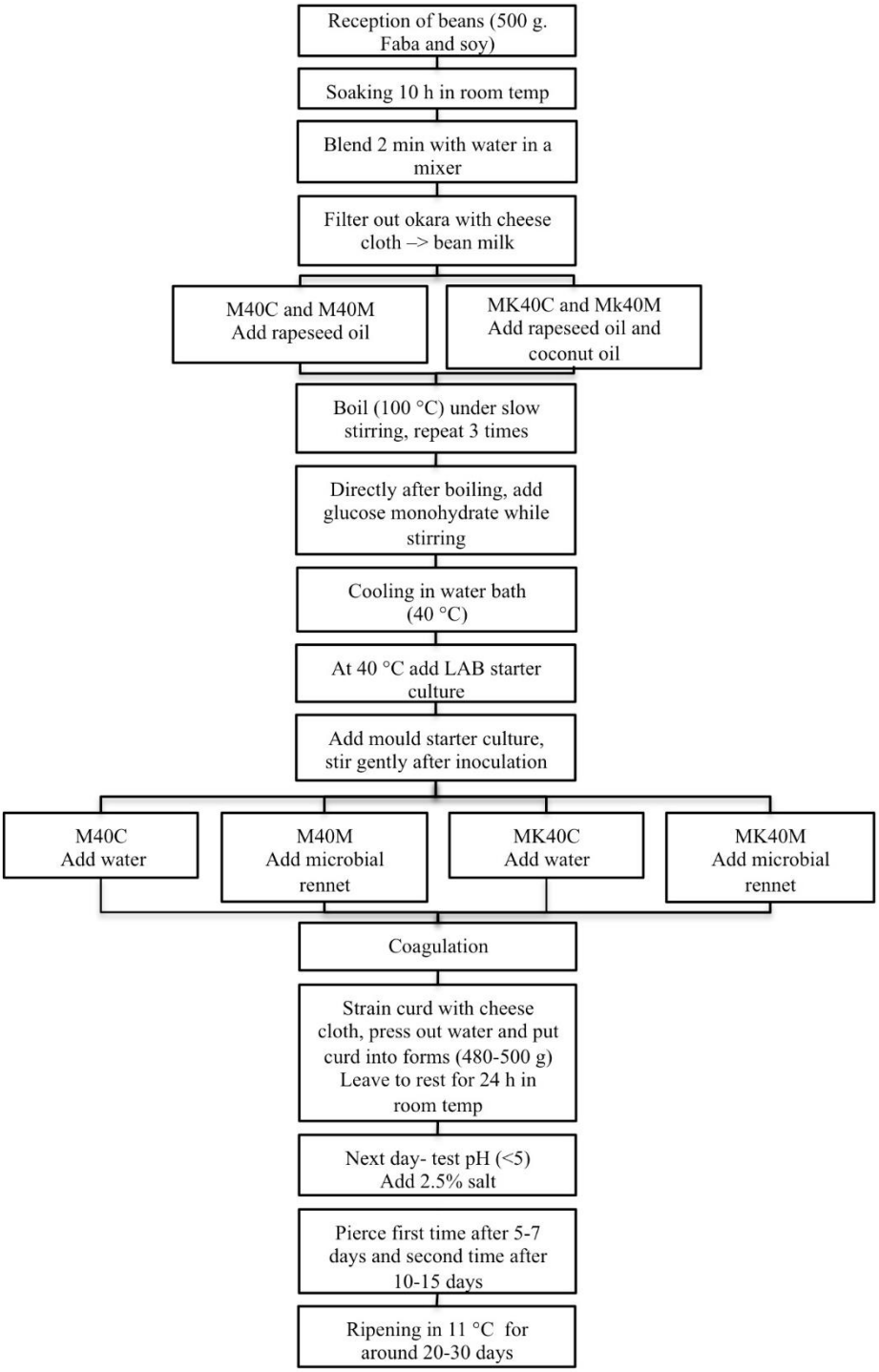

Figure 2. Flow chart

5 Results and discussion

5.1 Experimental

Bacteria

Results from the microbial analyses of blue-mould tofu from three different trials are summarized in Table 4.

Trial 1.

There was extensive growth of different bacteria on particularly BCSA VRBG and MSA plates. Unidentified species of *Enterobacteriaceae* and *Staphylococcus* were confirmed on VRBG and MSA plates by microscopy and confirmation tests. Colonies on VRBG plates appeared as dark purple and round shaped in differing sizes. In microscope could rod shaped bacteria be seen, which accord to the cell shape of Enterobacteriaceae. Gram test on a colony from VRBG indicated positive results, which means that the bacteria present was gram negative. Oxidase test showed a negative result. Results from the confirmation tests indicates that there could be a growth of Enterobacteriaceae, though the colony morphology was not typical for *E. coli* and the media allows growth for different species therefore the presence of *E. coli* excluded. Referring to the NMKL method No. 144, typical colonies of *E. coli* are pink or red coloured on VRBG.

On MSA plates, round, shiny, white/yellowish colonies could be seen. Many small cocci shaped bacteria, some in aggregates and some alone were seen in the microscope, which indicated with great probability presence of *Staphylococcus*. However, no distinct pH change was observed, which is detected as a change in color from red to yellow on agar. This indicated an absence of *S. aureus*, since the bacteria are mannitol fermenting (Adams & Moss, 252-253). Results from confirmation tests showed that bacteria present were gram positive and oxidase negative, which shows a probability of *Staphylococcus* growth.

One suspected *B. cereus* colony from a BCSA plate 6 (10^{-3}) was observed. Under the microscope, many rod-shaped bacteria could be seen. The rods were bound together in chains of different lengths or appeared alone. Many spores could be clearly distinguished. The colony morphology and colour appeared as typical for *B. cereus.* Typical colonies on BCSA are large, have a distinct bluish color and are surrounded by an egg yolk precipitate of the same color, as described in manufacturer's instruction. *B. cereus* was counted to levels of 10^3 cfu/g.

BGA plates showed growth of few small, white/yellow and wet colonies with a yellow zone around on several plates. Colonies on XLD had a similar appearance. The growth of *Salmonella* could be excluded since the colony morphology did not match with typical morphology for *Salmonella.* Colonies typical for *Salmonella* appear black on XLD and pink on BGA, which is described in the manufacturer's instructions.

Trial 2.

There was no identified *S. aureus*, *E. coli* or *Salmonella* in the cheese from second trial. There was either any growth of any bacteria on BP, XLD and BGA plates. A few small, round and dark purple colonies were observed on some VRBG plates. The colony morphology did not correlate with typical colonies of *E. coli*, the appearance was the same as in trial 1, therefore could presence of *E. coli* be excluded. One suspected colony on plate BCSA 9 (10^{-1}) was after re-streaking and microscopy identified as *B. cereus*, with regards to the colony morphology and cell appearance. As in trial 1, the morphology of the colony on BCSA was very typical for *B. cereus*, see description in section above. Colonies on blood agar were large, white/grey, irregular with a distinct haemolytic zone, which is typical for *B. cereus* on blood agar, according to the NMKL method No. 67. The enterotoxin test showed a negative result which means that no toxins were produced, either haemolytic or non haemolytic enterotoxins. *B. cereus* was counted to levels of 10 cfu/g.

Trial 3.

There was no identified *S. aureus, Salmonella, B. cereus*, *E. coli* or *L. monocytegenes* in the cheese in the third trial. Different colonies were present on XLD, BGA, VRBG and BCAB. Colony appearance was similar to the colonies from trial 1, however the growth was not as extensive. Some round brown/grey colonies with a thin opaque zone around were detected on BP plates. The colonies were examined under microscope, and were assumed to be *Staphylococcus* due to cocci shape of the cells. However, no *S. aureus* were identified due to colony morphology that differed from with the typical appearance of *S. aureus* on BP. *S. aureus* colonies appears as grey-black and surrounded by a clear zone, as described in the manufacturer's instructions. BactiStaph agglutination test showed a negative result and presence of *S. aureus* could be excluded. However, Baird parker agar allows growth of other *Staphylococcus* species. On VRBG very small and dark purple colonies could be seen on several plates (the same appearance as the colonies in previous trials), which also were examined in the microscope, where rod-shaped cells were seen. These colonies might be *Enterobacteriaceae*, however none fit the typical morphology for *E. coli*. Some colonies were present on one ALOA plate (cheese 2) and PALCAM plate (cheese 4). *L. monocytogenes* could be excluded on PALCAM plate due to colony morphology (white with uneven edges) which was not typical for *L. monocytogenes*. Typical *L. monocytogenes* colonies appear surrounded by a distinct black halo on PALCAM plates, as described in the manufacturer's instructions. On ALOA green small, round and shiny colonies could be seen. This correlates with appearance of *L. monocytogenes*, although a surrounding halo could not be seen. Green/blue colonies with a haemolytic zone are typical for *L. monocytogenes* according to the NMKL method No. 136. Either could the haemolysis be detected on blood agar, presence of *L. monocytogenes* could then be excluded. Growth of *L. monocytogenes* is inhibited at pH values below 5.5, though growth can in some cases occur down to pH 4.4 (Adams & Moss 2014, 226). pH of the blue-mould tofu is controlled to a value ≤ pH 5.0, which can be seen as a

important factor to inhibit growth of *L. monocytogenes*, as well as other bacteria. *L. monocytogenes* is able to form biofilms on the equipment, therefore regularly sanitation and cleaning of the equipment are also important measures (Kousta et al., 2010).

Table 4. *Summary of results from the three cheese trials*

Trial	***S. aureus***	***Salmonella***	***E. coli***	***B. cereus***	***L. monocytogenes***
1	Not detected	Not detected	Not detected	Detected	-
2	Not detected	Not detected	Not detected	Detected	-
3	Not detected	Not detected	Not detected	Not detected	Not detected

Moulds and yeasts

No moulds were identified neither in the bean milk nor in the beans. Some chloramphenicol resistant bacteria were present on a few plates of bean milk samples. The colonies appeared as small, white and round, yeast-like on the plates. By microscope rod shaped bacteria could be identified and yeast growth excluded. The absence of growth on the plates with soy and faba beans is most likely because of the treatment with herbicides and fungicides on beans to avoid infections in the field. Since moulds and yeasts are considered to be eliminated in the product during heat treatment the risk for them to appear in the product in small, however good hygiene is important to avoid contamination during production (Mavropoulos & Arvanitoyannis, 1999). Good ventilation in the handling and storage facilities is an important factor to avoid contamination by air, since spores are spread in the environment, supported by the study made by Temelli et al. (2006). Relative humidity and temperature are important to control in the ripening room. Roquefort cheese is usually ripened in air temperature at 5–10 °C and relative humidity at 95% (Sandrou & Arvanitoyannis, 2000).

Food safety is, as mentioned earlier in this work, one of the most important factors to achieve in food production. The blue-mould tofu is a ready-to-eat product, making the food safety aspect even more important. Even though tofu and similar products are great media for microorganisms to grow (Rossi et al., 2016), no foodborne pathogens (except *B. cereus* at low counts) were present in the blue-mould tofu. However, a varying growth of unidentified, probably non-pathogenic bacteria could be seen on the plates. More bacterial growth on the first cheese compared to the second and third cheese depends most likely to a high degree on better hygiene during production at later stages. This result shows the great importance of proper hygiene during production and confirms the information from the literature (Kousta et al., 2010). According to Temelli et al. (2006) improved hygiene practice and hygiene training of personnel during production of cheese are necessary to minimize the risk for microbial contamination. Ashenafi (1994), state that

microbiological quality of tofu can be significantly improved by disinfection of hands, knife and cutting board, and also cleaning of the press after daily production.

In trial 2, the handling and hygiene measures were improved compared to cheese tested in trial 1. Plastic gloves where used during handling of cheese, the cheese clothes were machine washed at 60 °C, cheese forms and glass beakers were washed in hot water. The incubator was cleaned with alcohol twice before ripening, and workbenches were also cleaned (for summary see Table 5). It has been reported that personnel hands can contribute to an increased count of staphylococci. The cutting knife has also been reported as an important source of contamination, particularly of mesophilic bacteria, in previous studies (Temelli et al. 2006; Arvanitoyannis & Mavropoulos, 2000). The product has also good properties to avoid bacterial growth due to the content of salt, low pH and the cooking step of bean milk.

Table 5. *Differences in production properties between the three cheese trials*

Trial	Hygiene	Ripening (time & temp)
1	No gloves or sanitation	10 days, 11 °C
2	Use of gloves Cleaning and sanitation of incubator Washing of cloths, forms and beakers	20-30 days, 11 °C
3	The same as for trial 2	20-30 days, 11 °C

Since *B. cereus* was found only as one colony in samples at dilution 10^{-3} in trial 1, and 10^{-1} in trial 2, it can be assumed that the contamination occurred during laboratory work. The bacteria showed anyway no toxin production, and can be estimated to be of less concern in the product, as the infective dose of *B. cereus* is high and estimated to be between 10^{5}-10^{8} cells/g food (Adams & Moss, 187). As mentioned above, some batch variation was discovered between the cheeses since there was an overall greater bacterial growth on plates from trial 1 compared to plates from trial 2, even though the procedure and ingredients were the same. The growth in trial 3 was more extensive than in trial 2, although less extensive than in trial 1. It is important to keep in mind that several bacteria are always present in the environment, in the facilities and on personnel, and that a minimal deviation in the process can cause contamination of the product. This points to the importance of implementation of production and hygienic procedures that are consistently followed during the whole production process. Variation in hygienic quality of the raw materials can also be an explanation for different growth in different batches. It must also be taken into account that contamination could also occur during lab work. To get a more precise result, the

bacteria could be identified to ensure that no other pathogens were present, although this work was limited to selected foodborne pathogens.

Identified CCPs and control points in previous studies in cheese productions are pasteurization, control of raw materials and prevention of recontamination by e.g. temperature controls during storage. Hygiene, cleaning, addition of rennet and salt, and coagulum cutting are also described as CCPs **(**Mavropoulos & Arvanitoyannis, 1999; Arvanitoyannis & Mavropoulos, 2000). There is a lack of studies available regarding HACCP implementation in tofu production.

5.2 HACCP-plan

The identification of hazards and CCPs is based on observation of the cheese production, laboratory work and literature review. The hazard analysis (Table 6) and HACCP plan (Table 7) is focused on the microbial hazards and is not complete, but can be seen as a first draft since the product is, at the time of writing, in a development phase. The microbiological analyses form the basis for a validation, which can confirm that the production process proceeds under hygienic circumstances, and that the suggested HACCP plan is working. pH during the process is measured to <5 which is important to inhibit microbial growth, and a important factor to address during production, which is a step considered as a CCP. Addition of salt and proper boiling of the bean milk (3×), are also most likely contributory factors for the positive results. Boiling kills most of the microorganisms although spore-formers and toxins may survive (Adams & Moss, 64-65), which has to be kept in mind during the following production steps. Boiling of bean milk is considered as a CCP since it is an important step to eliminate microorganisms.

Another identified CCP is the receiving step of beans, a certificate that ensures that beans are free from unsafe levels of mycotoxins should be required in the reception of beans. Mycotoxins can survive the production steps, including cooking of bean milk (Kabak, 2009), which can be a danger in the final product. Therefore pH monitoring, boiling and reception of beans are considered as the main steps which ensure the product safety. The CCPs were chosen with regard to the fact that they are easily controlled and crucial for the safety of the product. Good hygiene, proper handling together with other PRPs ensures food safety in the remaining control points. Since *E. coli* and *Salmonella* origin mainly from humans or foods of animal origin (Prencipe et al., 2010), they can be considered as a low risk in the product.

Table 6. *Hazard analysis*

Processing step	Potential hazard(s)	Justification	Significance (yes/no)	Symptoms caused by hazard	Preventive measure(s)	CCP?
Reception of beans	Beans can contain air or soil borne pathogens as moulds, e.g. *Fusarium* and *Alternaria*	Moulds have been associated with contamination of beans in field and during storage - risk for mycotoxin production	Yes	Mycotoxicosis with varying symptoms	Require certificate that ensures that received beans are free from unsafe levels of mycotoxins	✓
	B. cereus	Widespread in the environment	Yes	Abdominal pain, diarrhoea and rectal tenesmus	Disinfection pH <5	
	Salmonella	Has been isolated from soybeans	Yes	Flu-like symptoms, vomiting, diarrhoea and abdominal pain	Boiling	
	Rocks and stones	Can “follow” from the field	Yes	Can cause damage on teeth and inner organs	Visual inspection of received beans	
Storage of received beans	Mould growth	Moulds can grow under improper storage conditions – risk for mycotoxin production	Yes	Mycotoxicosis with varying symptoms	Control humidity (≤ 70%) and temperature (10 to 25 °C) in storage facilities	
Mixing	Metal splinter	Can be released from knives in the mixer if stones or other hard material are present among received beans	No	Can cause damage on inner organs	Visual inspection of received beans for stones and hard materials	
Boiling of bean milk (100 °C)	Some pathogens/spores may survive during insufficient heat treatment	Mycotoxins and bacterial spores (e.g. *B. cereus*) survives during heat treatment	Yes	Intoxication with varying symptoms	Temperature control Regular microbiological analysis for moulds and spore forming bacteria	✓
Cooling to 40 °C	Spore forming pathogens such as *B. cereus*	Spore forming pathogens can start to grow if the cooling proceeds over a long period	Yes	Abdominal pain, diarrhoea and rectal tenesmus	Cool in water bath Reach 40 °C within 1 hour	
Handling of beans, milk and curd	*S. aureus*	Present on the skin	Yes	Vomiting, nausea, stomach cramps, retching, prostration and diarrhoea	Disinfection, cleaning of equipment and facilities, use gloves during production	

	E. coli	Faecal bacteria, can be present on skin	No	Symptoms in gastrointestinal tract e.g. diarrhoea	Cooking, use gloves during production, handwashing, heat treatment	
	Salmonella	Present in food of animal origin or infected humans	No	Flu-like symptoms, vomiting, diarrhoea and abdominal pain	Pasteurization of bean milk, cooking, use gloves and handwashing	
Addition of microbial rennet	Poor coagulation can lead to impaired syneresis-> increased risk for microbial contamination Addition of microbial rennet at too high temperatures result in a hard coagulum	Quality of the enzyme preparation can vary	Yes		Temperature control (40 °C) Visual control of coagulation	
Cutting Filtering Pressing Forming	Contamination from equipment By e.g. *L. monocytogenes*	Can form biofilms Growth can continue during ripening and storage since the bacteria can grow in refrigerator temperatures	Yes	Flu-like symptoms, can be harmful for pregnant and immunocomprised	Disinfection and cleaning of equipment and hands Cooking	
pH control <5	Risk for growth of spore forming pathogens as *B. cereus*	Spore forming pathogens may survive the cooking Risk for growth if pH drop is not sufficient	Yes	Sickness with symptoms depending on microorganism	Add LAB starter culture at 40 °C	✓
Piercing with needle	Contamination with pathogens as *S. aureus* or moulds	Present on the skin and environment	Yes	Vomiting, nausea, stomach cramps, retching, prostration and diarrhoea	Disinfection of needle Use gloves	
Ripening (11 °C)	Moulds	Present in the air	Yes	Varying symptoms	Regularly cleaning and disinfection Control humidity (70-80%) and temperature (11 °C) in incubator	

Table 7. *HACCP plan*

CCP	Potential hazard(s)	Critical limits	Corrective actions	Monitoring	Verification	Documentation
Reception of beans	Mycotoxin production by moulds as *Fusarium* and *Alternaria*	Mycotoxins below legislated limits for cereal products Certification from supplier must be presented	Rejection of lot and/or change supplier if the certification is not received	Control of certification that ensures that received beans are free from unsafe levels of mycotoxins	Control of certification Analysis of random samples	Operator log book
Boiling (100 °C)	Some pathogens may survive during insufficient heat treatment	100 °C	Repeat heat treatment	Visual control that at least three bubbles are breaking the surface of milk every second. Can be complemented with temperature measure.	Check temperature and procedure (bubbles) logs Calibration of thermometer	Operator logs visual control of bubbles and temperatures
pH control	Growth of spore forming pathogens e.g. *B. cereus*	pH <5	Discard batch if pH >5 after 24 h	Production worker measure pH of the curd from each batch after 24 h rest, and note values in pH log	Calibration of the pH meter pH logs checked by the manager	Operator logs pH and calibration

6 Summary & conclusions

It emerged that the blue-mould tofu has a high hygienic status. The appearance of growth of possible *Staphylococcus, Enterobacteriaceae* and other unidentified bacteria depends on the fact that bacteria are spread in the environment and in the raw products. No food pathogens were identified except low counts of *B. cereus* in trial 1 and 2 respectively, however the bacteria were not toxin producing and the contamination occurred most likely during laboratory work. The differences in amount of bacterial growth between trials one and two, that had improved hygienic measures during production confirms that hygiene is very important during production to be sure that the food produced is safe to consume. This study has focused on specific microorganisms and the microbiological analyses were performed on cheese from three cheese production trials. More studies should be done on the product, especially in the final commercial scale production environment, to establish a good food safety management system. It is important to keep in mind that the production is performed on a research level and that the production and environmental properties will change in large-scale production. Storage and packaging are also important to evaluate when developing a HACCP plan.

Identified CCPs in the production are receiving of beans, boiling of bean milk and pH monitoring. Receiving of beans is controlled by requirement of a certificate that received beans are free from unsafe levels of mycotoxins. Boiling is controlled by visual control that at least three bubbles are breaking the surface every second, and temperature controls. The pH is controlled to be <5. As a conclusion, the blue-mould tofu is based on this work regarded to be a product with good hygienic properties. Good hygienic measures during production, cooking of bean milk, pH <5 and reception of certified beans will decrease the risk for contamination and thus make the blue-mould tofu safe to consume.

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Appendix 1. Media recipes

XLD agar
33 g agar
600 ml H_2O

Heated in microwave oven, do not autoclave.

Baird-parker agar base
28 g agar
400 ml H_2O
4 vials RPF supplement

Autoclave at 121 °C for 15 min. Let cool to 48 °C.
RPF supplement: add 10 ml H_2O to each ampoule with supplement. 1 ampoule/ 100 ml dH_2O. Mix gently. When the agar has reached 48 °C, add 4 ampoules and mix well.

Brilliant green phenol –red lactose sucrose agar
30.9 g agar
600 ml H_2O

Not autoclaved.

Brain heart agar
20.8 g agar
600 ml H_2O

Autoclave at 121 °C for 15 min.

Salmonella Rappaport enrichment
20.9 g enrichment
500 ml H_2O

Autoclave at 121 °C for 15 min.

Bacillus cereus agar base
34.5 g agar
800 ml H_2O
Polymyxin B
Egg yolk

Autoclave at 121 °C for 15 min.
When cooled add 0.476 ml Polymyxin B and 40 ml egg yolk, mix thoroughly.

Violet red bile glucose agar
30.8 g agar

800 ml H_2O

Heat in microwave oven until everything is dissolved, do not autoclave.

Malt extract agar
19.2 g agar
400 ml H_2O
0.04 g Chloramphenicol

Autoclave at 121 °C for 15 min.

PALCAM agar base
35.4 g agar
500 ml H_20
1 vial PALCAM supplement

Autoclave at 121 °C for 15 min.
Dissolve 1 vial of PALCAM supplement with 5 ml dH_20.
Add to cooled (45-50 °C) medium and mix.

ALOA agar
35.3 g agar
500 ml H_20
1 vial ALOA enrichment supplement
1 vial ALOA selective supplement

Autoclave at 121 °C for 15 min. Dissolve ALOA enrichment supplement with 5 ml dH_20. Pre-warm ALOA selective supplement to 48-50 °C. Add supplements to cooled (45-50 °C) medium and mix.

Fraser broth
Full strength
28.7 g broth
500 ml H_20
1 vial Fraser selective supplement

Autoclave at 121 °C for 15 min. Add 1 vial of Fraser selective supplement dissolved with 5 ml H_20 to room tempered broth and mix.

Half strength
12.9 g broth
225 ml H_20
1 vial Fraser half selective supplement

Autoclave at 121 °C for 15 min. Add 1 vial of Fraser half selective supplement dissolved in 3 ml H_20 to room tempered broth and mix.

Peptone water

10 g Peptone
5 g Sodium chloride
1L H_2O
pH: 7.2 ± 0.2

Autoclave at 121 °C for 15 min.

Buffered peptone water
10 g Peptone
5 g Sodium chloride
3.5 g disodium phosphate
1.5 g Potassium dihydrogen phosphate
pH: 7.2 ± 0.2

Autoclave at 121 °C for 15 min.

Nutrient agar, Blood agar and Mannitol salt agar were received as ready made.

Appendix 2. Cheese recipes

Cheeses analysed in trial 1.

Cheese nr. and type	Faba	Soy	Glucose	Rape-seed oil	Coconut oil	LAB starter culture	Mould starter culture	Microbial rennet
2: CK C	+	+	+			+	+	
1: CK M	+	+	+			+	+	+
3: R20 C	+	+	+	++		+	+	
5: R30 C	+	+	+	+++++		+	+	
6: R40 C	+	+	+	++++++		+	+	
7: R40 M	+	+	+	++++++		+	+	+
8: RK20 C	+	+	+	+	+	+	+	
9: RK20 M	+	+	+	+	+	+	+	+
10: RK30 C	+	+	+	++	++	+	+	
11: RK40 C	+	+	+	+++	+++	+	+	
12: RK40 M	+	+	+	++++	++++	+	+	+

C: no microbial rennet
M: with microbial rennet

R20, R30 - R means rapsolja
RK - rapsolja and kokosolja
C - control
20 - target 20% fat in cheese
30 - target 30% in cheese

Cheeses analysed in trial 2.

Cheese nr/type	Faba	Soy	Glucose	Rape-seed oil	Coconut oil	LAB starter culture	Mould starter culture	Microbial rennet
1: M40C 5-1	+	+	+	+++++		+	+	
5: M40M 5-1	+	+	+	+++++		+	+	+
9: MK40C 5-1	+	+	+	++	+++	+	+	
13: MK40M 5-1	+	+	+	++	+++	+	+	+

C: without microbial rennet
M: with microbial rennet
M40C - M means matolja
MK40C - MK means matolja + coconut oil

Cheese analysed in trial 3.

Cheese nr/type	Faba	Soy	Glucose	Rape-seed oil	Coconut oil	LAB starter culture	Mould starter culture	Microbial rennet
1: M40C 5-3	+	+	+	+++++		+	+	
2: M40M 5-3	+	+	+	+++++		+	+	+
3: MK40M 5-3	+	+	+	++	+++	+	+	+
4: MK40C 5-3	+	+	+	++	+++	+	+	

Appendix 3. Popular summary

Microbiological analysis and implementation of a food safety plan for a new vegan product

The demand for vegan foods is increasing, together with the fact that consumers requires foods with high quality, long shelf-life and that are at last but not least safe to consume. How can the food producer ensure that the products are safe to consume?

The European Commission has established several food legislations, applicable for all food producers and handlers. There are several systems that food producers can implement in their productions to ensure that the food is safe to consume. One of those systems is the HACCP (Hazard analysis critical control point) methodology, where hazards are identified in the production, and in a next step a plan is implemented with aim to control and prevent the hazards to occur.

A new vegan product is under development at SLU (Swedish University of Agricultural Science), a blue-mould tofu. The product is made by Swedish soy and faba beans and has similar properties to a blue-mould cheese. The production procedure can be compared to regular tofu, except the addition of mould, which characterizes the blue-mould tofu. Aim of this study was to evaluate whether the product is safe to consume.

The blue-mould tofu is in this study analyzed with respect to microbiological pathogens in lab. Pathogens can be of concern for the food safety aspect, since many of them can cause foodborne diseases. A HACCP plan has also been implemented, based on a literature review and the laboratory work. Several microbial hazards have been identified as, *Staphylococcus aureus, Bacillus cereus, Salmonella, Escherichia coli* and *Listeria monocotogenes*. These bacteria can enter the product in raw materials or during production and cause foodborne diseases. Moulds as *Aspergillus* and *Fusarium* can spoil beans, and also be present in the production environment, which should be kept in mind during production.

Results showed that the product has a high hygienic quality, which means that it is a safe product, since no pathogens were identified in the product. Important factors to control and implement during production are cleaning of equipment and facilities, personal hygiene, boiling of bean milk, pH measure, salt addition and receiving a certificate together with beans which ensures that received beans do not contain

mycotoxins, which are produced by moulds. If these steps are controlled, then the product will maintain a high food safety status. Identified critical control points in the HACCP plan are receiving of beans, boiling of bean milk and pH measure. pH should be kept <5, to inhibit growth of pathogens.