Product development of side-stream press cake through fermentation

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Abstract

People in the EU are increasingly turning towards eating more plant-based foods with a lower climate impact than meat and dairy products. The subsequent increase in the production of plant-based products has resulted in large volumes of side-stream press cake, which up until now has mainly been used for animal fodder. The aim of this project was to develop a safe food product for human consumption, made through fermentation of Swedish oat and fava bean press cakes. To achieve this, the microbial safety of the raw press cakes was analysed, and different methods of fermentation with both lactic acid bacteria (LAB) and *Rhizopus oligosporus* were investigated. The fermented press cake was assessed according to different evaluation criteria and microbial analysis. The finished product was an oat press cake tempeh that is safe for human consumption. This product could be sold as a healthy, protein and fibre rich alternative to meat. The possibility of transforming a low value by-product into a high value food product would also be profitable for plant-based companies, and lead to a great reduction of waste in the whole industry.

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Abbreviations

GRAS	–	Generally recognized as safe
LAB	–	Lactic acid bacteria
LOD	–	Limit of detection
LOQ	–	Limit of quantification
MC	–	Moisture content
PDCAAS	–	Protein Digestibility Corrected Amino Acid Score
RH	–	Relative humidity
VRBD	–	Violet red bile dextrose

1 Introduction

People in the EU are increasingly turning towards plant-based products due to concerns about climate change and for health reasons. The EU-funded Smart Protein Project, which was conducted in 11 EU-countries between 2018-2020, showed that total sales in plant-based products increased by 49% in two years. Spurred by this massive growth, food companies are now developing and releasing more plant-based products at a record rate (Plant-based foods in Europe: How big is the market? 2021).

While Sweden was not part of the Smart Protein Project, the plant-based sector in Sweden is experiencing similar growth. According to a survey about the eating habits of Swedish people, the number of vegetarians and vegans has been steadily increasing in the past 6 years. Additionally, the proportion of people who opt for vegetarian options 2-6 days every week has increased from 19% to 30%. Sales of plant-based frozen foods in grocery stores, which includes products such as vegan sausages and plant-based minced meats, has been steadily increasing by about 10-15% every year. The refrigerated section, which includes products such as oat and bean milk, has experienced an even greater growth of 20-40% per year (Vegobarometern, 2022).

This massive new demand from consumers for production of plant-based products has led to a drastic increase in the volume of side streams. A quite common side stream for plant-based companies is the press cake left over after production of oat or bean milk. The press cake is rich in fibre, protein and nutrients but is typically used for animal feed or even outright discarded as it spoils very quickly (Funeteg and Jonasson, 2022). Companies with large side-streams of press cake, such as the Swedish companies The Green Dairy AB and Oatly, are attempting to find new, environmentally friendly, and economic usages. Fermenting the residue from side streams is a way to accomplish this. (Production Residues, 2020, The Green Dairy, 2022).

Press cake from soybeans, also called okara, is a side stream from the production of soy milk and tofu which is created in large quantities in parts of Asia. While okara is mainly used for livestock feed or discarded, it can be fermented which may enhance health and nutritional benefits. In Indonesia, the soy okara is fermented with specific strains of Rhizopus mould to create tempeh, a white fluffy solid cake where the internal structure is created by the mycelia during the fermentation process. This product with high rated sensory qualities is perfect for many dishes and is a staple protein source in Indonesia. Not only is it possible to create tempeh from a wide variety of legumes, including fava beans, but it is also possible to ferment cereals into tempeh (Shurtleff and Aoyagi, 1979). The fermentation of press cake made from oats seems to be novel. No literature was found about this topic.

Recent experiments have shown that fermenting soy okara with Lactic acid bacteria (LAB) may even produce a meat analogue by adding *Lactobacillus* spp to plant-based matrices. (Razavizadeh et al., 2021).

The benefits of fermentation are plentiful. Fermentation processes can create products that have good sensory properties for human consumption, even from side-streams that otherwise might be discarded. It can enhance the health benefits and nutritional qualities of okara by microbiological degradation of enzyme inhibitors that hinder digestion and oligosaccharides that can cause flatulence. LAB fermentation lowers the pH of the food, inhibiting the growth of spoilage and

pathogenic organisms and simultaneously increasing the shelf-life of the product. Fermentation may also improve the flavour and palatability of the press cake which opens a path to product development for humans (Vong and Liu, 2016).

This project aims to create a safe and edible fermented product from the side-streams of Swedish vegan dairy companies.

2 Aims

The aims of the project are:

- To investigate microbial safety of the press cakes made of oats and fava beans before and after fermentation.
- To develop a process through which press cakes can be fermented with LAB or *Rhizopus oligosporus* and made into a food product.

3 Theoretical background

3.1 Composition of the raw press cakes

The press cake is the residue that is left over after production of vegetable milks. The production of vegetable milks like soy, fava and oat milk are very similar. The basic process is that the raw legume or cereal is soaked in water and then ground. Thereafter there is a separation process in which the liquid is separated from the solid matter (ProVeg, 2019).

Press cake composition data

The raw press cakes from fava bean, soybean and oat used in the thesis project is supplied from The Green Dairy. Analytical composition for protein, fat, carbohydrates, and fibres in wet basis for the press cakes in the project are shown in Table 1 (Funeteg and Jonasson, 2022).

Table 1: The analytical components of the press cake supplied by The Green Dairy.

Analytical components	**Soybean okara**	**Oat press cake**	**Fava bean press cake**
Protein (%)	9.0	10.3	8.0
Fat (%)	3.0	0.7	0.5
Carbohydrate (%)	1.0	9.8	4.1
Fibre (%)	9.0	9.0	17.7

Proteins

The protein that these press cakes contain is not equal nutritionally and there is variable quality in protein digestibility and amino acid coverage. The Protein Digestibility Corrected Amino Acid Score (PDCAAS) is a measurement for the amino acid protein composition and digestibility. Animal based proteins are complete and have a score at or very near 1.00. Plant proteins typically have a lower PDCAAS score as they frequently lack certain amino acids. Fava bean for instance lacks methionine and cysteine, while lysine is limited in oat. Soy protein has a PDCAAS score of 0.99, the highest of any plant, comparable to beef with 1.0. The PDCAAS score does not necessarily mean that the protein quality or amino acid coverage is bad, additional protein from other sources must be included in the diet (Hertzler, et al., 2020).

Carbohydrates

The carbohydrate content, meaning the sugars and starch (excluding dietary fibre), is quite limited in the legume press cakes, especially in soy okara. The oat press cake has a high content of carbohydrates, even higher than the fibre content. This may improve fermentation as the lack of carbohydrates may limit microbial growth (Vong and Liu, 2016).

Fibres

The press cakes are filled with dietary fibres. These fibres are divided into soluble and insoluble fibres, both being resistant to human digestive enzymes. The insoluble fibres are hard for gut bacteria to ferment and help promote healthy bowel movement. The soluble fibre fraction is digested by gut bacteria and promotes the growth of bacteria beneficial to health. Oats are rich in soluble β-glucans, while fava bean and soybean have a more mixed fibre composition. Too high fibre intake is associated with flatulence, stomach aches and diarrhoea or constipation (Makki, Deehan, Walter and Bäckhed, 2018).

Antinutritional factors

Apart from the health promoting aspects of the composition of the press cakes, there are also compounds present within the foods that have a negative impact on the body's absorption of nutrients. It is important to consider and shape the production process so that the levels of these antinutritional factors are minimized.

Phytates are antinutritional factors that binds strongly to metal ions, iron, zinc, calcium etc which forms complexes that cannot be absorbed, thereby reducing nutritional content. It is very common and found in soy, fava, and oats. Soaking in water is a method used to reduce phytic acid concentration (Multari, Stewart and Russell, 2015).

Trypsin inhibitors are proteins that reduce the activity of trypsin, an important digestive enzyme. They are mainly present in legumes, with particularly high concentrations in soybean protein, and may be removed by heat treatment. (Vong and Liu, 2016).

Tannins are compounds that reduce the bioavailability of proteins and minerals in the food. They are considered a main antinutritional factor in fava beans but are present in a very wide range of foods. Similar to phytic acids, the concentration can be reduced by soaking (Multari, Stewart and Russell, 2015).

Vicine and convicine are compounds found in fava beans that is toxic to people affected by hereditary loss of an enzyme. This is called favism and it causes haemolytic anaemia. This antinutrient can be removed by soaking or heat treatment, or a combination of the two (Multari, Stewart and Russell, 2015).

3.2 Fermentation of press cake

One way to increase the value of the side stream press cake is fermentation. Not only can antinutritional factors readily be metabolised by microorganisms, but also other macromolecules which can further improve the nutritional quality, and potential health benefits of the finished fermented product. This can be done by a wide range of microbes, including mould, bacteria, and yeasts.

The process of fermenting soybeans and soy okara have spread all around the world, but are most popular in Asian countries like China, Japan, and Indonesia. Soybeans are fermented to, for example, produce natto with *Bacillus natto*, tempeh with *Rhizopus* spp, and soy sauce with *Aspergillus* spp (Liu, 2008).

For this thesis project, the fermentation of the press cakes was limited to starter cultures containing lactic acid bacteria (LAB), and *Rhizopus oligosporus*.

Lactic acid bacteria fermentation

Lactic acid bacteria are a large group of bacteria that produce lactic acid as a residual product after metabolism of carbohydrates, which is a requirement for growth. Most LAB are generally recognized as safe (GRAS) and greatly improve flavour, shelf-life, safety and even offer health benefits. They are used in fermentation of products like sauerkraut, cheese, salami, sourdough bread and yoghurts that are enjoyed all around the world (Bintsis, 2018).

Since LAB produce lactic acid as a metabolism by-product, the effect that they have on the food matrix is that the pH is lowered. These bacteria are acid tolerant and can survive and thrive in pH that other microorganisms cannot. Not only that, but they also produce bacteriocins that inhibits growth of other microorganisms. This is beneficial for the safety and shelf-life aspects, as there are few pathogens or spoilage microbes that can survive the harsh environment (Narvhus and Axelsson, 2003).

Many LAB have beneficial health effects when ingested. Some of their probiotic effects may include protecting against infection by food borne pathogens, stimulate the immune system and controlling cholesterol levels. Additionally, fermentation with LAB may reduce content of some antinutrients, such as phytates and trypsin inhibitors (Rašić, 2003).

LAB are used to safely ferment many different foods into healthy, nutritious food products with good sensory qualities. For these reasons LAB starter cultures seem promising for fermentation of soybean, fava bean and oat press cakes.

Rhizopus oligosporus fermentation

Rhizopus oligosporus is an aerobic fast growing filamentous fungus that creates three dimensional mycelia networks on solid substrate. These strands of mycelia can bind the substrate into firm, tightly bound cakes also known as tempeh.

Tempeh is a traditional food from Indonesia typically made from whole soybeans but can be made from a wide range of other legumes and even cereals like wheat and rye. It is also possible to create tempeh from press cake residues. Soy press cake is widely used for this purpose, but other legume press cakes, like fava bean, have also been used. (Shurtleff and Aoyagi, 1979).

An effort to create tempeh from rapeseed press cake with *R. oligosporus* is currently underway to utilise this valuable side stream. Unlike other common moulds, *R. oligosporus* does not produce any mycotoxins harmful to humans and are considered GRAS (Lücke et al., 2019). The fungal fermentation removes phytates and other antinutritional factors from the press cake while simultaneously producing some vitamins and increasing free amino acids. *R. oligosporus* produces riboflavin (B_2), niacin (B_3) and small amounts of carotenoids. In some tempeh, vitamin B_{12} can be present, but this is created by other bacteria. (Nout and Kiers, 2005). Tempeh made from oat press cake does not seem to have any references in literature but has been made from whole oats (Aaslyng and Højer, 2021).

The preferred growth conditions of *R. oligosporus* are temperatures between 25-41.5 °C, with an optimum growth at 37 °C and pH 5.5. During fermentation the mould generates its own heat and temperatures up to 49 °C have been shown by natural fermentation. This high temperature may inhibit the growth of or kill the *R. oligosporus.* Tempeh fermentation is usually performed at 30-32 °C for 18-72 hours. The mould needs high relative humidity for growth, around 70% (Ahnan-Winarno et al., 2021).

R. oligosporus is acid tolerant and can survive in pH as low as 3.5, but the low pH will inhibit growth severely and limit mycelia formation. Additionally, fermentation with *R. oligosporus* produces ammonia which progressively raises the pH of the substrate. Because of this overripe tempeh may have a strong smell of ammonia (Ahnan-Winarno et al., 2021).

3.3 Microbiological risks

The main organisms of microbiological concern studied for this thesis are *Bacillus cereus,* a common soil bacterium and various *Enterobacteriaceae.*

Bacillus cereus

B. cereus is a common soil bacteria found almost everywhere. It is a facultatively anaerobic spore-forming gram-positive bacterium. Some strains of *B. cereus* may produce toxins that cause foodborne illness. This bacterium is thought to be responsible for up to 12% of all food borne illnesses worldwide, second only to *Salmonella* infections in prevalence (European Food Safety Authority, 2018).

B. cereus has a variety of strains that may be present and cause illness in a wide variety of foods, such as cooked meats, vegetables, cereals, milk products and rice (Tallent, 2020). The contamination of *B. cereus* is highly relevant to this project, as it is of high concern for the vegan dairy industry. This is exemplified by the recall of a batch of Oatly Oatmeal 1 litre in Finland 2022, where 2 cases of illness and 29 complaints were recorded (Varjonen, 2022).

The bacterium has optimal growth temperatures at 28-35 °C, with a minimum and maximum growth temperature at 4 °C and 48 °C respectively. The pH range that *Bacillus* spp can grow at is 4.9-9.3 pH (Tallent, 2011). The spore forming nature of the bacteria make them difficult to eliminate through industrial processes.

There are two main types of toxins that can be produced that cause two distinct illnesses, one emetic variant and one diarrheal form. The emetic illness is caused by a toxin called cereulide, which is a cytotoxin that is highly resistant to heat, acid, and digestive enzymes, making it very difficult to inactivate or remove from contaminated food. It is also very small and has no immunogen properties, making it difficult to detect. Since the toxin is so stable, no viable cells need to be present for intoxication. *B. cereus* in concentrations of log 3- log 5 /g food may be enough to produce disease-provoking levels of cereulide. The onset of disease is up to 6 hours after consumption, and lasts 6-24 hours (Dietrich et al., 2021). However, not all strains of *B.*

cereus have the gene for expression of cereulide, and even high concentrations of *B. cereus* may in some cases be safe to eat (Christiansson, 2008.).

The second illness, caused by enterotoxin, produced symptoms of diarrhoea and abdominal cramps. The enterotoxin is, compared to cereulide, not very stable. This means that the toxin content is drastically reduced by cooking and digestion in the stomach. Therefore, most cases of this illness require *Bacillus cereus* to colonise the gut to produce symptoms. These occur within 8-16 hours of consumption of contaminated food. The infective dose is around 10^5-10^8 CFU/g (Dietrich et al., 2021).

In this project *B. cereus* was chosen as a suitable candidate for microbial safety evaluation due to it being a common contaminant in this type of food product.

Enterobacteriaceae

Enterobacteriaceae is a large family of gram-negative bacteria, including over 100 species. They are common gut bacteria and food spoilage microorganisms and can affect food product quality. There are pathogenic species that may be dangerous, like *Salmonella enterica* in this family. The majority are not, however, classified as pathogenic, but they are a good indicator of microbial safety. *Enterobacteriaceae* are quite common contaminants where food hygiene and sanitation protocols are unsatisfactory (Baylis, 2006.).

The *Enterobacteriaceae* are monitored throughout the thesis project due to the concerns mentioned above.

4 Materials and methods

4.1 Raw materials and cultures

Raw press cake made from oat, fava bean and soybean were supplied by The Green Dairy in Karlshamn, Sweden. The oat and soy press cake are taken directly from current process lines while the fava bean press cake is done batchwise in pilot scale. The date that the oat and soy press cakes were taken from the decanter and the when the fava bean press cake batches were produced are reported in Table 2. There may be variation in the composition of the press cakes depending on what. The press cakes were stored frozen at -18 °C and vacuum sealed in plastic bags at Kemicentrum, Lund. The weight of the frozen bags varied between 400g - 2200g.

Table 2: The date that the press cakes were produced and sampled from The Green Dairy.

Fava Bean	2021-02-19
Fava Bean	2022-01-27
Soybean	2019-08-08
Oat	2019-08-08

The yoghurt fermentation cultures used in the project were supplied by Danisco. The origin and names of the cultures are shown in Table 3.

Table 3: Product name, bacterial composition and product origin for yoghurt starter cultures.

Product name	Bacterial species	Place of origin
Danisco® VEGE 033	*Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*	Copenhagen, Denmark
Danisco® VEGE 053	*Lactobacillus bulgaricus, Streptococcus thermophilus, Lactobacillus lactis, Lactobacillus acidophilus and Bifidobacterium lactis.*	Copenhagen, Denmark

The concentration starter culture used was:

- 0.1 g Danisco Vege 033 / 10g press cake.
- 0.23 g Danisco Vege 053 / 10g vegetable press cake.

The tempeh starter cultures were bought online from Njordstorps Permakultur Gård and Humlegårdens ekolager. The origin of the starter culture and company purchased from are shown in Table 4.

Table 4: The companies that supplied the tempeh starter and the origin of the starter.

Tempeh starter	Njordstorps Permakultur Gård Njordstorp, Sweden
Tempeh starter	Humlegårdens ekolager Zoersel, Belgium

Bacillus ChromoSelect agar is a medium that selects for *Bacillus* spp. The different species show up as coloured colonies by which they can be analysed. A polymyxin-B sulphate supplement can be added which isolates *B. Cereus* and *B. Thuringiensis* as blue colonies and inhibits growth of other *Bacillus* species (*Bacillus* ChromoSelect Agar, 2022).

Violet red bile dextrose (VRBD) agar is a medium that selects for *Enterobacteriaceae* (GranuCult® VRBD (Violet Red Bile Dextrose) agar, 2022). The agar used in the project are shown in Table 5 below.

Table 5: The name of the agar used for the project, by which company it is produced and place of origin.

Bacillus ChromoSelect agar	Merck, Germany
Violet red bile dextrose agar	Merck, Germany

4.2 Microbial evaluation of raw materials

It is very important to know whether there are high concentrations of bacteria or moulds in the press cake prior to fermentation. Toxins produced by these organisms in the raw material can continue into the product. Since the raw materials used for the project have been considered a side-stream not intended for human use, the same regulations as those intended for human use do not apply. A microbial investigation of the raw material, the oat, fava, and soybean press cake were performed. The effect of a heat treatment on the microbial content was evaluated by plating the press cakes after boiling.

Included in this microbial analysis were the raw samples themselves, the raw samples after 20 minutes of boiling in water at 100 °C for 20 minutes (boiled samples) and cooled down to room temperature before usage. The samples were diluted by mixing 1g of sample into autoclaved dilution tubes with 9 ml of peptone (bacteriological peptone + 0.85 %NaCl) and vortexed until mixed. Dilution series were constructed to the desired dilution factor by diluting into peptone.

For the microbial investigation, samples were plated on three different agars, VRBD, *Bacillus* ChromoSelect, and *Bacillus* ChromoSelect with polymyxin. The samples were plated with

different dilutions. 100 µl of the diluted fluid was applied to the agar plate with an automatic pipet, and homogeneously distributed around the agar using autoclaved glass beads.

Both boiled and raw samples were plated on double plates with triplicates. The dilution scheme used for the raw and boiled samples is shown in Table 6 below.

Table 6: Dilution factors used in raw samples and boiled samples plated on different agar.

Agar type	Raw sample dilution factor	Boiled sample dilution factor
VRBD	10	10
Bacillus ChromoSelect	10^4-10^5	10
Bacillus ChromoSelect + polymyxin	10	10

The samples on VRBD plates were incubated for 24h at 37 °C aerobically, and samples on the two *Bacillus* agars, both with and without polymyxin, were incubated for 48h at 30 °C aerobically. The same incubation conditions were used throughout the entire project.

After incubation a colony count of the plates was performed. For the VRBD agar, all colonies were counted. For the plates with *Bacillus* ChromoSelect agar, only green and blue colonies are counted. Colonies coloured green indicate *B. subtilis*, which is a common spoilage microorganism. Blue coloured colonies indicate *B. cereus* or *B. thuringiensis* as the two are biochemically identical (*Bacillus* ChromoSelect Agar, 2018). Since the plate method used cannot differentiate between the two, all blue colonies are considered potentially pathogenic *Bacillus cereus* in this project.

The recording of the plate count was done according to the FDA Bacteriological analytical manual. The acceptable range of CFU/plate that is used for this project is 25-250 colonies per plate. Too few colonies lead to high standard deviations and too many leads to cluttering, where colonies compete for growth and may be difficult to count. Plates with colonies under that range were reported as less than limit of quantification (LOQ), which can be calculated through the following formula: LOQ = 25 x 1/d where d is the dilution factor. The limit of detection (LOD) for colony count is 1 CFU/plate. Samples in which all triplicate plates showed no colonies can be recorded as <LOD. For plates with a colony count above the accepted range, the colonies were recorded as too numerous to count (TNTC) and displayed as >LOQ in the tables in the project (Maturin, 2001). This way of recording the plate count was used throughout the project.

4.3 Development with yoghurt cultures

The fermentation of the three different starting materials, oat- and fava bean press cake and soy okara was done using the same method. 50 g boiled press cake was inoculated with the desired concentration of vegetable yoghurt starter culture and mixed thoroughly in a small beaker. The inoculated sample was then put into a 50 ml tube for incubation. The tube was sealed with parafilm to allow the fermentation to progress anaerobically and incubated at 30 °C for 24h. Triplicate tubes were used for each type of boiled press cake and for each inoculum.

After incubation of the fermented samples, they were plated with the same agar and method used previously for the raw material. The pH was also measured after fermentation. The colonies were counted after the plates had been incubated.

4.4 Development of press cake tempeh

Fermentation was performed on the oat press cake with the mould *Rhizopus oligosporus*. Fermentations on the fava bean and soy press cakes were also done, but the focus of the thesis was the fermentation of oat press cake. Due to constraints in both time and material, the tempeh production process was not individually tailored for each type of press cake, and therefore they use the same method for tempeh production.

The process of fermenting tempeh from the press cake can be divided into smaller steps. There are fewer process steps needed for the fermentation of the press cake side-stream compared to when fermenting tempeh from whole beans, as the press cake is already processed. The steps used to produce press cake tempeh are heat treatment, inoculation, bag preparation and incubation. The process steps used to ferment tempeh were overviewed, and the production process was changed and improved continually throughout the project by trial and error. A flowchart showing the general production process for press cake tempeh is shown in Figure 1.

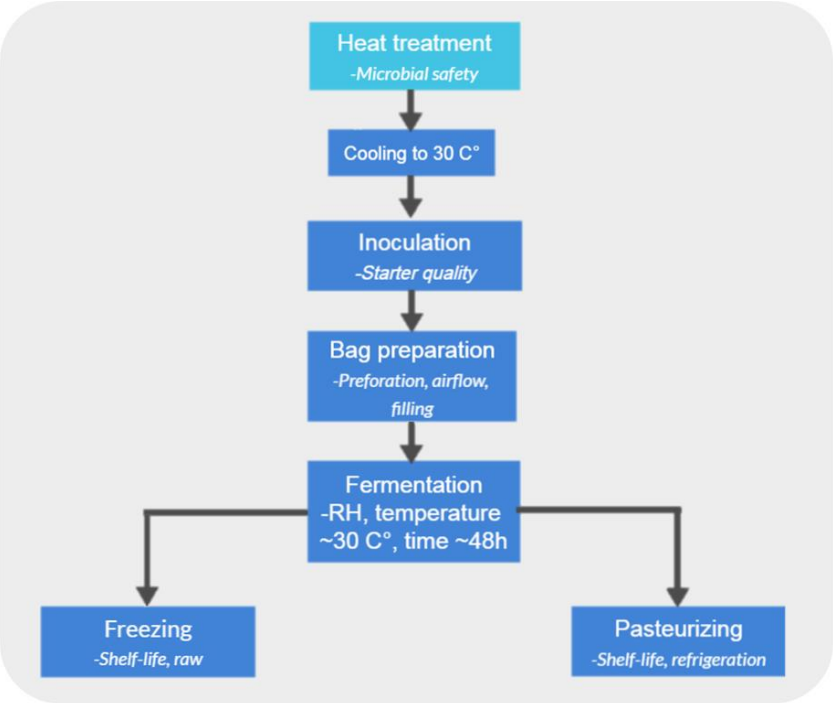

Figure 1: Flowchart depicting the press cake tempeh production process.

For the first three batches of tempeh the process was done as try-outs to determine whether the mould can grow in the press cakes, and to try out different methods and conditions of incubation. These batches were done to assess the method for making press cake tempeh and to make some adjustments, and to see which parameters could be tweaked. The lab protocol and methods used for these batches are noted in appendix A.

Heat treatment

The bags of press cakes were taken out of the freezer room and defrosted, either by putting the bags in the refrigerator the day before or defrosting the bags in water.

For the first batches of tempeh, the bags were opened, and the contents put into water and then boiled for 30 minutes. A timer was started when the water reached boiling temperatures. After boiling, the boiled press cake was strained through a thin mesh and pressed to remove as much water as possible and left to cool. For some trials, the boiled press cake was dried in an oven at 60 °C for 1 hour or heated on a hob for 30 minutes to evaporate water.

This idea was discarded in favour of instead boiling the bags directly, similar to sous vide. This was done as it was found that the moisture content of the material is essential for the formation of the mould cake. Using the vacuum bags in this way keeps the moisture content at a constant level. The boiling time was initially set to 30 minutes but tripled later in the project to reduce bacterial count.

After boiling, the press cake was allowed to cool down to below 30°C. At this point samples of 10 grams were collected for dry matter analysis in small plastic sample tubes before continuing with the next step in the process.

Inoculation

The boiled material was fermented by inoculation with commercial tempeh starters. Initially the starter culture used was from Njordstorps Permakultur gård, but due to running out of that starter, a culture ordered from Humlegårdens ekolager was used for a few trials until a replacement culture from Njordstorps Permakultur gård arrived. The concentration of tempeh starter culture that was used for the inoculation was 3 g starter culture / kg substrate.

The boiled press cake to be inoculated was put into a large bowl and weighed. In the trials where different pH was tried, vinegar was added at this point, prior to inoculation. The amount vinegar added depends on the desired pH. This was determined in an experiment where different volumes of vinegar were added to the press cakes and then the pH was measured. The starter culture was weighed up according to the mass of the boiled press cake and then rigorously mixed by hand for more than 2 minutes. The press cake was always controlled to be under 30 °C before inoculation.

Bag preparation

To assist in making a solid shape of the finished cake, perforated polyethylene bags were used. The plastic bags used in the project for the tempeh fermentation are 0.4 l Ziplock bags. The bags are perforated by hand with a fresh toothpick with 1 mm thickness to create a grid with evenly spaced holes. Both sides of the bag were punctured simultaneously.

The bags were then filled with the inoculated press cake. The amount of material that was inserted ranged from 150 - 200 g. The bags were then pressed on lightly to even out the bags into square cakes of uniform height before being put into the incubator. In general, triplicate bags were used, but in some cases, there was not enough raw material left, in which case only duplicate bags were used.

Incubation

There were two different incubators used for the project. The main one used for most fermentations is a Termaks B8260 Lab Incubator (Kungsbacka, Sweden) A blast freezer, Nordtech plus (Plus 5, Fontanafredda, Italy) was also used as an incubator in a few trials, even though it might sound counterintuitive. The blast freezer can be set to temperatures up to 90 °C and relative humidity up to 70%.

Due to concerns about the contamination of the incubator and the potential for mould spreading, an alternate fermentation chamber was used initially. This consisted of a plastic box with an

airtight lid where the filled Ziplock bags could be placed together with a bowl of water inside the incubator. Approximately two bags could fit inside each such box.

The fermentation chamber method was later discarded in favour of placing the filled Ziplock bags on top of a baking sheet over a metal grate inside the incubator.

The incubators were set to 30 °C for 48 h. The relative humidity in the blast freezer was set to 70% during the trials it was used. To monitor the temperature and relative humidity (RH) of the incubator for the duration of the fermentation, an RH & Temperature logger (Reed, ST-171, Wilmington, USA) was placed inside the incubator. This device was not used inside the fermentation chambers themselves, however.

After fermentation, the tempeh was taken out of the incubator, evaluated based on the four criteria, samples taken for microbial analysis and then either chilled or frozen.

4.5 Evaluation and analysis for press cake tempeh

Quality evaluation

Based on the knowledge gained from the first batches, evaluation criteria were established and used to guide the work forward. The next step of product development was to conduct trials with the purpose of improving the consistency and quality of the tempeh.

Fermented tempeh was evaluated based on four criteria: Firmness, coverage, smell, and visual appearance. The criteria are defined below:

- Firmness is the overall feel. The tempeh should be able to be lifted as a cohesive cake and thin slices or cubes should hold together without crumbling.
- Coverage is how much of the surface area is covered by white mycelia. The coverage should be close to 100%, without empty spots.
- The smell of the tempeh should be pleasant, a little sweet or mushroom aroma. Some smell of ammonia is acceptable, but it should not be overpowering. Any unpleasant smell should be noted as it may be indicative of spoilage.
- Visual appearance is the colouring. Tempeh with some black sporulation near the pinholes is okay, but too much should be avoided. Other colours such as reds and yellows are undesirable and are indicative of contamination.

The safety and quality aspects of the tempeh are intrinsically interlinked, as the microbial fermentation can result in a delicious filling product, or an outright hazard. As such, it is important to investigate both the microbial parameters and the process itself to create a good, consistent product.

Dry Matter analysis

For the process, the dry matter of the boiled vegetable press cake was quantified throughout the project to gain a better understanding of how moisture content affects the mould fermentation and mycelia formation. The samples were taken after the heat treatment step detailed previously.

The dry matter analysis was conducted by weighing small metal cups, and then adding 3 g of sample. The metal plate cups were placed in an oven at 103 °C for 24 hours. Thereafter, the metal cups were placed in a desiccator for 30 minutes and then weighed. The dry matter analysis was performed in triplicates.

The equation used for calculation of the moisture content (MC) is shown in eq.1 below where the *w* is the wet weight and *d* is the dry weight of the sample:

$$MC = \frac{w-d}{w} \times 100 \quad (1)$$

Microbial analysis

Once a reproducible method of creating tempeh that meets the evaluation criteria was developed, the microbial safety investigation was launched.

This part included plating tempeh samples on agar plates using the same methods as previously in the project. The dilution used was dependent on projected bacterial concentrations. The finished oat tempeh was plated.

The starter cultures used were also plated to check for sources of microbial contamination. The starter cultures were plated on VRBD and *Bacillus* ChromoSelect and counted.

Bacillus cereus enterotoxin detection

To check for the presence of *Bacillus cereus* enterotoxin present in the food, a toxin assay kit was purchased. The toxin assay was done on the three raw press cakes, and on the finished tempeh from trials 6 & 7. The lab manual for this toxin detection kit is available from the manufacturer (BCET-RPLA TOXIN DETECTION KIT, Thermofischer Scientific, Boston, USA)

The raw material and two batches of finished tempeh were analysed using the enterotoxin detection kit. Each sample has two rows of the plate designated. The first is an enterotoxin assay and the second is an agglutination control. The last well in a row must be negative or the results are considered invalid. Interpretation of the results was done by comparison to Figure 2. The sensitivity in detecting the enterotoxin is 2 ng/ml in the test extract.

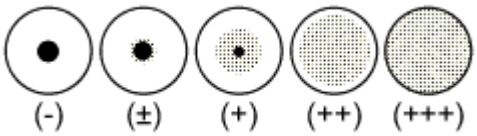

Figure 2: A schema showing interpretation of agglutination results.

5 Results and discussion

5.1 Microbial evaluation of raw material

The microbial analysis of the raw material and the effect of subsequent heat treatment on aerobic plate count was conducted. The focus areas for the microbial count were *Enterobacteriaceae* spp and *Bacillus* spp.

There were no *Enterobacteriaceae* colonies present on the VRBD agar for the raw oat and soy press cake. This means that the viable count is below the limit of detection (LOD) of 1 CFU/plate. When adjusting for the dilution factor this results in <100 CFU/g press cake. Only the fava bean press cake samples had a few *Enterobacteriaceae* colonies but were not in the limit of quantification (LOQ). All colony counts on boiled press cake were <LOD. The results from the plate count on VRBD agar of the raw press cake are shown in Table 7.

Table 7: The results for the aerobic plate count on VRBD agar of both boiled and raw samples. The numbers are reported as CFU/g samples.

Type	Fava bean press cake (CFU/g)	Oat press cake (CFU/g)	Soy okara (CFU/g)
Raw	<2500	<100	<100
Boiled	<100	<100	<100

This result is not very surprising, as the press cake is taken from the decanter at around 80°C which should eliminate the heat sensitive *Enterobacteriaceae* (Baylis, 2006). These results show that the raw press cake was handled in a way which did not contaminate it with *Enterobacteriaceae*, during sampling, transport, and handling for the preparation of samples.

The plate count from the *Bacillus* ChromoSelect agar is shown in Table 8. The results show that the concentration of *B. subtilis* is <log 5 CFU/g for the raw samples, and under <250 CFU/g for the boiled samples. LOQ for the raw samples was $2.5*10^5$ because a higher dilution factor was used for the plates.

Table 8: The results for the aerobic plate count on *Bacillus* ChromoSelect agar of both boiled and raw samples. The numbers are reported as CFU/g samples.

Type	Fava bean press cake (CFU/g)	Oat press cake (CFU/g)	Soy okara (CFU/g)
Raw	$<2.5*10^5$	$<10^5$	$<10^5$
Boiled	$<2.5*10^3$	$<2.5*10^3$	<100

Lower concentrations of *B. subtilis* than expected were found on the raw material for this plate count. The expected concentrations were based on plate results from previous work, where *Bacillus subtilis* count was around log 5 for soy, oat, and fava bean press cake under the same conditions (data not published). It may be that different batches of raw material contain highly variable bacterial content, or that the bacteria are not uniformly spread out in the material.

Bacillus ChromoSelect agar with added polymyxin-B should, according to the producer, inhibit the growth of *Bacillus subtilis*, and thus there should be very few green colonies present. This was not the case, as the raw fava sample had green colonies present at concentrations above the limit of quantification. There were no blue colonies (*B. cereus)* on the fava bean plates. This may not necessarily be because there is no *B. cereus* present, but rather that they were outcompeted on the plates.

The plated soy okara was completely free from any colonies for all plates, which means that the bacterial content was below the LOD of 100 CFU/g.

For the raw oat sample however, both blue and green colonies were present, indicating the presence of both *B. cereus*, and *B. subtilis*. Four plates had green colonies and two plates had blue colonies. Plate counts inside the LOQ range were recorded and the averages are shown in Table 9 for the green colonies and Table 10 for the blue colonies.

Table 9: Plate count results for *Bacillus* ChromoSelect agar with added polymyxin-B on the raw and boiled material. This table shows the results for green colonies.

Type	Fava bean press cake (CFU/g)	Oat press cake (CFU/g)	Soy okara (CFU/g)
Raw	$>2.5*10^5$	$1.2*10^4$	<100
Boiled	<100	<100	<100

Table 10: Plate count results for *Bacillus* ChromoSelect agar with added polymyxin-B on the raw and boiled material. This table shows the results for blue colonies.

Type	Fava bean press cake (CFU/g)	Oat press cake (CFU/g)	Soy okara (CFU/g)
Raw	<100	$3*10^3$	<100
Boiled	<100	<100	<100

The high number of *B. subtilis* colonies indicates that the polymyxin-B sulphate supplement used does not inhibit *B. subtilis* enough. This could hinder enumeration of *B. cereus*. Polymyxin-B interacts with lipopolysaccharides, which gram-positive bacteria, like *Bacillus* spp, do not possess (Zavascki et al., 2007). It will however reduce background microflora by elimination of gram-negative species, which is very useful for the accuracy of the colony count.

Microbial analysis showed that there are high levels of *Bacillus* spp in the raw fava bean press cake and moderate levels of both *B. subtilis* and *B. cereus* in the raw oat samples, even after heat treatment in the factory. The concentration of *B. cereus* on some of the agar plates was worrying as high levels of cereulide could be produced in the raw press cake and remain later in production. However, a more thorough investigation of the *B. cereus* content of the oat press cake needs to be performed as the polymyxin supplement for the *Bacillus* ChromoSelect agar does not seem to be a very reliable method for quantification of *B. cereus*.

Even with a low certainty of the plate count, the big impact of heat treatment on colony growth was shown. After boiling there was a clear reduction to below LOD of *B. subtilis* in the fava bean press cake and reduction of both *B. subtilis* and *B. cereus* to below LOD in the oat samples. The raw soy okara already had CFU/g below LOD, which indicates safe levels of microorganisms.

5.2 Development with yoghurt cultures

After fermentation of three types of press cakes with the two yoghurt cultures, the fermentation was evaluated by pH measurement and plating.

The results of the yoghurt culture fermentation were promising. Immediately after breaking the seal on the fermentation tubes, they released a scent reminiscent of yoghurt. This was the case for all three vegetable press cakes for both starter cultures. It was not possible to distinguish the scent of the different starter cultures from each other. The yoghurt smell from the oat fermentation was a little less pronounced compared to the fermented beans.

The consistency of the fermented soy and fava beans was smooth and creamy. The oat on the other hand had more chunky bits inside, very reminiscent of porridge. The taste was like yoghurt but more acidic.

The pH that is desirable to completely inhibit the growth of *B. cereus* is 4.9, which is the case with all the samples for both yoghurt cultures. The results from the pH measurements are shown in Table 11 below.

Table 11: The average pH of different press cakes fermented with the raw material and the two Danisco starter cultures. The pH was measured after 24h.

Type	Fava bean press cake	Oat press cake	Soy okara
Raw sample	6.35	6.55	6.65
Danisco® VEGE 033	4.3	4.50	4.25
Danisco® VEGE 053	4.0	4.25	4.50

While the pH results are very promising, the inhibition of growth does not mean that the food is safe, as it may contain bacterial spores. Thus, the bacterial colony count is very important to monitor the bacterial content.

The VRBD plates did not contain any colonies, regardless of press cake or starter culture used. This was expected since the boiled press cake was used and that the yoghurt cultures lowering the pH will inhibit and kill bacteria. The results for the VRBD plate count are shown below in Table 12. Since no colonies were present on any plates the *Enterobacteriaceae* count is below the LOD.

Table 12: The VRBD plate count for the two yoghurt starter cultures.

Type	Fava bean press cake (CFU/g)	Oat press cake (CFU/g)	Soy okara (CFU/g)
Danisco® VEGE 033	<100	<100	<100
Danisco® VEGE 053	<100	<100	<100

The fava bean and soy press cakes had few to no *Bacillus* colonies present on the *Bacillus* ChromoSelect plates. The oat press cake that was fermented with Danisco VEGE 033 had few colonies, but the oat sample fermented with Danisco VEGE 053 had an estimated count of $9*10^3$ *B. subtilis* present for both agars. No *B. cereus* colonies were present on any of the plates, meaning that the concentration is <LOD. The plate count results are shown in Table 13.

Table 13: The plate count results from the fermentation of boiled press cakes with the two yoghurt cultures on *Bacillus* ChromoSelect agar both with and without polymyxin-B supplement.

Bacillus ChromoSelect agar	Fava bean press cake (CFU/g)	Oat press cake (CFU/g)	Soy okara (CFU/g)
Danisco® VEGE 033	$<2.5*10^3$	$<2.5*10^3$	<100
Danisco® VEGE 053	$<2.5*10^3$	$9.0*10^3$	<100
+Polymyxin-B	Fava bean press cake (CFU/g)	Oat press cake (CFU/g)	Soy okara (CFU/g)
Danisco® VEGE 033	<100	<100	<100
Danisco® VEGE 053	<100	<100	<100

From a safety perspective, this batch of fermented food is not hazardous, owing to the low concentration of pathogens. *B. subtilis* is not associated with any diseases GRAS. A concentration of log 3 CFU/g *B. subtilis* present in the fermented oat press cakes is therefore safe to consume. At very high *B. subtilis* concentrations the quality of the food may be affected.

Fermenting press cakes with LAB cultures may be good for further development of products from the side streams of Swedish vegan dairy products. One potential product that springs to mind is a fermented bread spread. The idea to use LAB fermentation was not further explored in the project after fermentation with *Rhizopus Oligosporus* to create press cake tempeh was deemed to have a higher potential due to the ability of the mould to give a cohesive structure to the press cake.

5.3 Development of press cake tempeh

For tempeh fermentation there are many factors to consider making a consistent high-quality product. On the side of the press cake this includes proper heat treatment of the press cake during processing in the side-stream to reduce the count of microorganisms, hygienic handling of the press cake and equipment and safe storage conditions. For the fermentation itself a high-quality starter culture is required, control and monitoring of growth conditions and a robust system for microbial safety.

The plan for the tempeh product development process was to use the four evaluation criteria detailed in 4.4.1; Firmness, coverage, visual appearance, and smell to guide subsequent trials. For any product development process, it is important to be able to monitor progress and evaluate results in a more methodical fashion. The lab protocols for the trials are described in appendix B.

Product development is a time-consuming process, with plenty of paths to choose from that can be investigated. Much time was spent on trouble shooting what went wrong with some fermentation batches and trying to find solutions for these.

Initial batches and trials

The product development of the tempeh was divided into two parts, the initial batches, and the trials. The protocols for the batches and tempeh trials are available in appendix A.

In the initial batches, all three types of press cake were used, oat, soy, and fava bean for tempeh fermentation. Both the fava bean and oat press cake fermentation resulted in firm cakes that were mostly covered in white mycelia. The bottom parts of the tempeh were not completely covered due to limited airflow. A picture of the fava bean press cake tempeh from the first batch is shown in Figure 3 below.

Figure 3: A photograph of fava bean press cake tempeh from batch 1.

The try-out batches confirmed that it is indeed possible to ferment the press cakes using *Rhizopus oligosporus* to create a firm edible cake made of oat, soy, and fava bean with acceptable sensory qualities (according to the author). A visual inspection is sufficient to determine success or failure of the fermentation, which allowed for the creation of visual evaluation criteria to guide the work forward.

Fava bean tempeh was developed until the raw materials in storage were exhausted. A new batch of fava bean press cake was supplied, but it was much wetter than the previous batch (80% MC compared to 75%) and was grey instead of yellow in colour. This effectively put a stop to fava tempeh development. The fava bean tempeh from trials 3-4 using the initial batch of fava bean tempeh was successful, however.

The fava bean tempeh from these trials fulfilled all the evaluation criteria. The tempeh cakes were fully covered in mycelia with no discoloration, were firm (even after cooking), and had a pleasant earthy, mushroom aroma.

The fava bean tempeh was easily cut into cubes and fried. With the lack of materials, a wider safety investigation was not performed as it was with the oat tempeh.

Tempeh growth conditions

Tempeh fermentation with *R. oligosporus* is a well-developed process and there is an abundance of literature on the subject, especially on the growth on soybeans. Acceptable growth conditions of the mould seem to be the same in the press cakes as whole bean tempeh. The growth conditions used in the project, 30 °C for 48h in the incubator seem to be widely used to make tempeh.

Incubator settings do not necessarily reflect the actual growth conditions. The temperature and relative humidity were monitored in both the blast freezer and lab incubator. The blast freezer was set to 30°C and 70% RH, but the temperature and RH oscillated between 27-31 °C, 50-60% respectively for most of the fermentation. After 37h the relative humidity started dropping

considerably in the blast freezer. A graph showing the temperature and RH during the fermentation is shown in Figure 4.

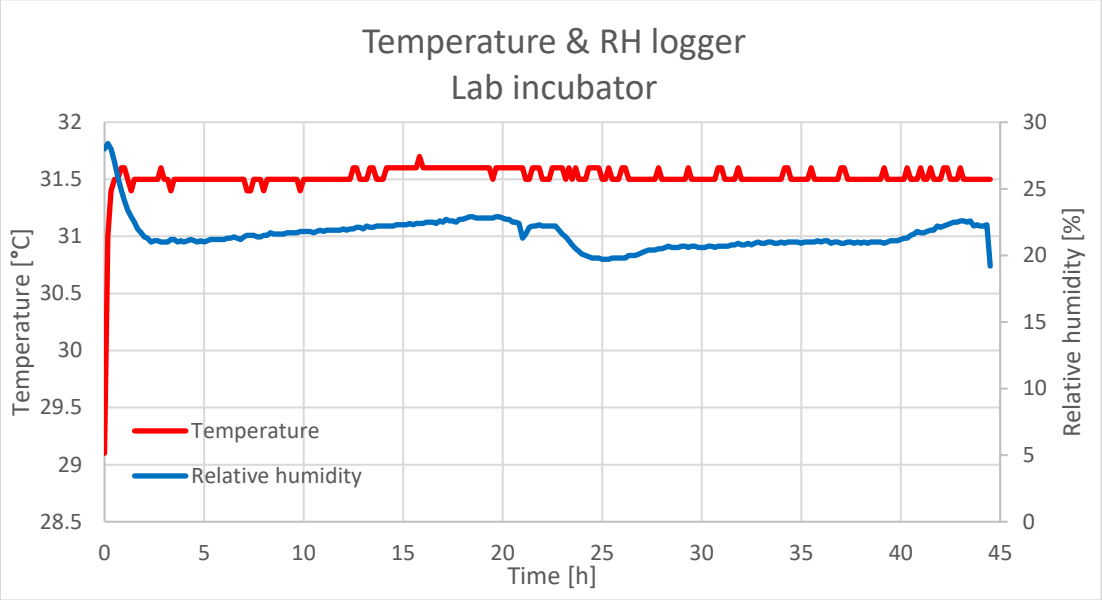

Figure 4: Temperature and RH logging of blast freezer during a fermentation trial.

The lab incubator temperature was more consistent, but instead of holding at 30 °C, the temperature was hovering around 31.5 °C. There were bowls of standing water inside the incubator which kept the relative humidity stable. A graph showing the temperature and the RH during fermentation is shown in Figure 5.

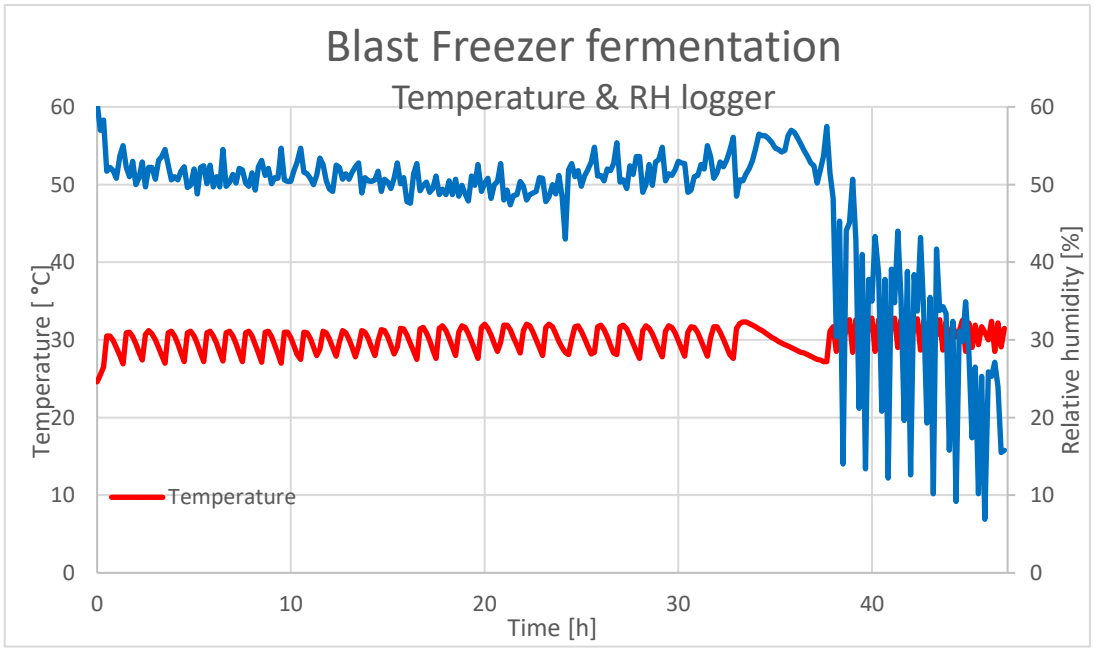

Figure 5: Temperature and RH logging of blast freezer during a fermentation trial.

There was no visually noticeable difference in the growth of *R. oligosporus* for either incubator during fermentation, even with the big difference in relative humidity. *R. oligosporus* needs a high humidity to grow but was still successful even in 20% RH. The reason for this may be that the local RH inside the polyethylene growing bag is higher than the outside air. During fermentation there were often condensation droplets hanging from the inside of the bag. These droplets may disrupt mycelia formation (Ahnan-Winarno et al., 2021).

5.4 Evaluation and analysis of oat tempeh

Quality evaluation

The oat press cake had a massive transformation after fermentation. In trials 4-7, there was substantial mycelial growth after just 24h. This indicates that the conditions for the growth of *R. oligosporus* are favourable, and possibly that the time of fermentation could be halved. The time of fermentation will have an impact on the taste and texture due to on-going microbial bio valorisation of the press cake. With further work done, the time of fermentation could be optimized, which would be a cost saving measure when scaling up production industrially.

When the pH was lowered there was a clear reduction in mycelia growth for pH 5.0, but the tempeh with pH 5.5 grew just as fast as in trials 4-6 that had no vinegar added. With consecutive fermentations fulfilling the evaluation criteria, the method and growth conditions used seem to work well. The mycelia fully cover the surface of the tempeh cakes with little discolouration and were firm (even after frying), and had a pleasant earthy, mushroom aroma. In Figure 6, a progression of the oat tempeh, from press cake to fried sample is shown.

Figure 6: Oat press cake (top left) prior to fermentation, and after 48h fermentation at 30°C (top right). Oat press cake tempeh in cubes (bottom left) and marinated & fried oat tempeh (bottom right). Pictures from trial 7.

R. oligosporus fermentation drastically changes the visual appearance and the texture of the oat press cake. Like the fava bean tempeh, the oat tempeh is firm, cohesive and, tasted mushroom-like. When marinated it is quite flavourful, with the frying giving it a crispy outside while retaining the mushroom texture in the middle.

Unlike the fava bean tempeh however, the oat tempeh does crumble a little bit. This may be due to the "chunks" of oats still within. A closeup picture of the oat tempeh structure is shown in Figure 7. This chunk of oat tempeh was ripped off and not cut to better show the structure. The mycelia are fully grown on the inside of the tempeh that binds the oat together. In the top right corner, there is some sporulating *R. oligosporus* visible.

Figure 7: Closeup of oat tempeh structure. Picture from trial 6.

Dry matter analysis

Based on the try-out batches and literature review, the moisture content was deemed an important factor to monitor. If the water content is too low, the water activity may decrease, which is crucial for production and activity of enzymes responsible for mycelial growth of *R. oligosporus* (Sarrette, et al, 1992). If it is too high, on the other hand the resulting tempeh is very wet and not cohesive, as shown in a few of the press cake tempeh batches. The risk of unwanted microbial growth is also increased (Shurtleff, W. and Aoyagi, A., 1979).

The dry matter content can be controlled in an industrial setting. The raw press cake was taken directly from the decanter after production. It may be possible to adjust the MC through different process parameters in the decanter or, if required, additional drying in a later process step. The decanter currently in use at The Green Dairy produced oat press cake with a MC at 57.2%. In Table 14 the moisture content from the oat press cake after boiling is shown.

Table 14: The moisture content of the oat press cake for the raw press cake and the boiled press cake used in the oat tempeh trials

Sample	Moisture content (%)
Raw Oat press cake	57.2
Trial 1	57.0
Trial 2	72.6
Trial 3	63.0
Trial 4	56.7
Trial 5	56.6
Trial 6 Mixed	54.5
Trial 6 Vacuum	57.0
Trial 7	55.0

Analysis of dry matter for oat was conducted for trials 1-7. The oat tempeh showed mycelia growth in trials at 63% but grew just as well at 57 %. When the MC of the oat press cake increased to 72.6 % the result was a foul-smelling disaster.

The dry matter analysis allowed for some identification of potential sources of error. A high moisture content may, for example, result in a more humid local environment inside the polyethylene growing bags which then forms condensation droplets. These droplets may disrupt the mycelia formation on the surface of the tempeh and result in a moist tempeh cake that does not fulfil the evaluation criteria.

Since the dry matter analysis takes 24 hours to perform, its main usefulness is not as a real-time quality control tool, but rather helping in finding sources of error or to help guide further optimization of growth conditions. Due to a wide range of factors in the tempeh-making process, it is difficult however, to use this method to pinpoint sources of error.

Microbial analysis

A microbial analysis of the finished tempeh from trials 5-7 was conducted to assess the safety, using the same method for the plate count as previously detailed.

From trial 5, plating of both the tempeh from oat and fava bean press cake was done on VRBD and *Bacillus* ChromoSelect agar. The fava bean tempeh from trial 5 did not meet the evaluation criteria, while the oat tempeh did. The fava bean tempeh was included in the microbiological evaluation out of interest to see if the cause of fermentation failure could be determined. The results of the plate count are shown in Table 15.

Table 15: Plate count results from trial 5 on VRBD and *Bacillus* ChromoSelect agar.

Trial 5	Fava bean 2022 tempeh (CFU/g)	Oat 2019 tempeh (CFU/g)
VRBD	$>10^4$	$>10^4$
Bacillus ChromoSelect	$>10^4$	N/A

The plate results for the Fava bean tempeh showed colony counts >LOQ for both VRBD and *Bacillus* ChromoSelect. This may help explain why this tempeh stank of overripened cheese and that the surface had little to no mycelia coverage.

The oat tempeh had a colony count of *Enterobacteriaceae* >LOQ. The *Bacillus* ChromoSelect plates were filled with the white mycelia of *R. oligosporus* which makes quantification of *Bacillus* spp impossible. The bottoms of the plates are covered in what looks like a smear of green and blue colonies. Pictures of a *Bacillus* ChromoSelect plate from the oat tempeh are shown in Figure 8.

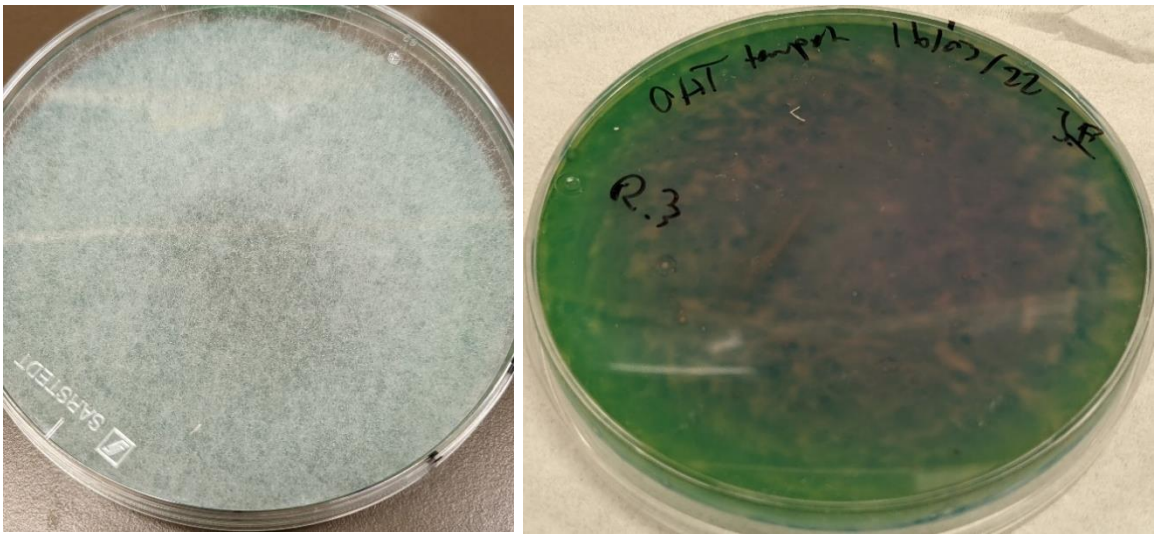

Figure 8: Oat tempeh from trial 5 plated on a plate of *Bacillus* ChromoSelect agar. Top of the plate (left) and bottom of plate (right) is shown.

The *Enterobacteriaceae* count was worrying and an investigation of probable sources of contamination was launched. It was speculated that the microbial contamination may have been from the tempeh starter cultures used. To check whether this was the case or not, the starter cultures from Humlegårdens ekolager and Njordstorps Permakultur Gård were plated.

There were no *Enterobacteriaceae* present on the VRBD plates for either starter culture which means that the concentration is <LOD of 100 CFU/g. The *Bacillus* ChromoSelect plates were plated on a dilution factor of 10^2 -10^5. The plates on 10^2 dilutions were filled with mycelia like in figure 5.4.2.1 (left), but without the green/blue colour on the bottom. A single green colony was observed on 1/3 plates on each starter culture. On 10^5 dilution factor Humlegården plates showed no mycelia growth, while the Njordstorps Permakultur Gård plates were partially covered. No bacterial colonies were observed on this dilution. The results from the plating of starter cultures on VRBD and *Bacillus* ChromoSelect plates are shown in Table 16 below.

Table 16: Table showing the results from the plating of the two tempeh starter cultures on VRBD and *Bacillus* ChromoSelect agar.

Starter culture plating	Njordstorps Permakultur Gård (CFU/g)	Humlegårdens ekolager (CFU/g)
VRBD	<100	<100
Bacillus ChromoSelect	$<2.5*10^3$	$<2.5*10^3$

The results from the plates suggest that neither starter culture is the source of contamination. The starter culture from Njordstorps Permakultur Gård has a higher concentration of viable *R. oligosporus* and will therefore be used for the next trials.

To reduce the colony counts and get a clearer understanding of the microbiological content of the tempeh, a tripled boiling time was introduced for the next trials. Boiling the bags in water may not provide adequate heating to the entire bag as the outer layers in contact with the bag surface get warmer than in the middle of the bag. This heat gradient present in the bags may have led to higher bacterial count.

Additionally, the lab incubator was cleaned and then disinfected with ethanol (70%). All kitchen equipment and surfaces used were also cleaned and then disinfected with ethanol (70%) instead of just washing with detergent.

In trial 6, a plating of the boiled press cake was performed, and two different boiling methods were evaluated. One where the content of the bag was mixed every 20 minutes and another where no mixing was done. The internal temperature was measure after mixing with an IR thermometer. The internal temperature never reached 100 C°, but stayed at over 85 C°**,** for over 50 minutes.

The new boiling methods showed a clear difference in the colony count for the *Bacillus* ChromoSelect plates. The colony count was reduced from 290 CFU/g (a mix of blue and green colonies) to below 100 CFU/g by mixing the content of the bag, which allows for more even heat distribution. *Bacillus* spores are not heat sensitive but can be reduced in number through heat treatment. The *Enterobacteriaceae* count was <LOD. A plate count of the new boiling methods is shown in table 5.4.2.3.

Table 17: Plate count results after boiling Oat press cake for 1.5 h. The effect of mixing the press cake was evaluated.

Trial 6	Oat press cake Boiled (CFU/g)	Oat press cake Boiled & Mixed (CFU/g)
VRBD	<100	<100
Bacillus ChromoSelect	290	<100

Fermentation in trial 6 resulted in nice firm cakes completely covered in mycelia when vinegar was not added for both boiling methods. This tempeh fulfilled the evaluation criteria. The tempeh from press cake where the pH was adjusted to 4.5 did not experience mycelia growth. Samples using both the mixed and unmixed boiling method were plated with and without added vinegar. The results from the plating of aforementioned samples are shown in Table 18.

Table 18: The plate results of fermented samples from trial 6 on VRBD and *Bacillus* ChromoSelect agar. The *Bacillus* colony count of the oat tempeh (mixed boiling) on *Bacillus* ChromoSelect has an asterisk* due to the plates having plenty of non-*Bacillus* colonies.

Trial 6	Oat tempeh (mixed boiling) (CFU/g)	Oat tempeh (CFU/g)	Oat tempeh 4.5 pH (CFU/g)	Oat tempeh 4.5 pH (mixed boiling) (CFU/g)
VRBD	$>10^3$	$>10^3$	<100	<100
Bacillus ChromoSelect	<100*	$>10^5$	<250	<100

There was a clear difference of *Enterobacteriaceae* colony count between vinegar and no vinegar samples. The sample with pH 4.5 showed no colonies, while the oat tempeh had over the far above the LOQ. Since there were no *Enterobacteriaceae* colonies detected after boiling, there must be contamination during the production process. Adjusting pH to 4.5 was too harsh for the mycelia to grow, however. Some *R. oligosporus* spores did survive alongside a smear of other microorganisms. This invalidated the lowest dilution plates due to low readability.

The results for the *Bacillus* ChromoSelect for the oat tempeh was very interesting. The unmixed oat tempeh showed numbers of *B. subtilis* over log 5, but no *B. cereus* colonies.
Instead of showing green/blue colonies for the mixed boiling oat tempeh there were mainly grey milky colonies or, for one triplicate a mix between grey milky and yellow instead. Figure 9 shows *Bacillus* ChromoSelect plates from the plating of the oat tempeh from trial 6.

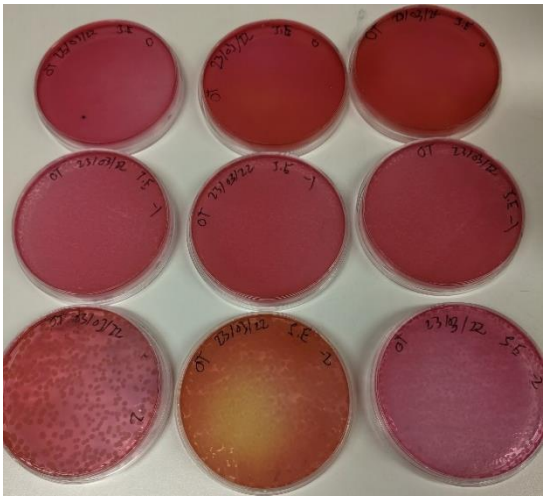

Figure 9: A photograph showing oat tempeh from trial 6 plated on *Bacillus* Chromo select agar with total dilution factor ranging from 10^1-10^3.

The colonies found on this tempeh are suspected to be *R. oligosporus* as some of the plates started to grow white mycelia. This is shown in Figure 10.

Figure 4: Mycelial growth from one of the plates of oat tempeh plated on *Bacillus* Chromo select agar

These results seem to indicate that the concentration of *Bacillus* species is very low as the typical colourful colonies are not present. This seems to suggest that the mixed boiling method was effective in removing *Bacillus* spp. Therefore, it may be possible to have tempeh that is safe from the spore forming bacteria by just using heat treatment. This would entail high hygiene requirements on the part of the producing company to avoid contamination.

As pH 4.5 did not produce a satisfactory product as there was no mycelia growth, a wider range of pH were tried in trial 7 on the finished oat tempeh; 4.75, 5.0 and 5,5. The growth of *B. cereus* should not be possible at all below 4.9 pH, but spores formed previously in the food may still survive to grow when the pH increases (Tallent, 2011).

The CFU/g *Enterobacteriaceae* on VRBD was $6*10^4$ for the oat tempeh with pH 5.5. When the pH was decreased even further, the count was <LOD. The plate results on the VRBD are shown below in Table 19.

Table 19: Plate results from VRBD plating of Trial 7.

Trial 7	Oat tempeh pH 5.5 (CFU/g)	Oat tempeh 5.0 pH (CFU/g)	Oat tempeh 4.75 pH (CFU/g)
VRBD	$6*10^4$	<100	<100

On the *Bacillus* ChromoSelect plates from trial 7 there was a lot of smearing and unclear plates, as mycelia growth makes count of colonies difficult. This is especially the case for the oat tempeh with pH 5.0, where only the 10^4 dilution plates were valid. The colonies on the 10^4

dilution plates were below the limit of quantification. The bacterial content of these tempeh samples should be lower than the bacterial concentration found in oat tempeh with pH 5.5.

For pH 5.5 both *B. cereus* and *B. subtilis* colonies were observed, at $2*10^3$ CFU/g and $6*10^3$ CFU/g respectively.

The tempeh with pH 4.75 showed barely any mycelia growth owing to the harsh conditions, and the bacterial colony counts are likewise very low. Both total colony count, and *B. cereus* count of the oat tempeh from trial 7 is shown in Table 20.

Table 20: Plate results from plating oat tempeh on *Bacillus* ChromoSelect agar. Both total colony count, and *B. cereus* count is shown below.

Trial 7	Oat tempeh 5.5 pH (CFU/g)	Oat tempeh 5.0 pH (CFU/g)	Oat tempeh 4.75 pH (CFU/g)
Total colony count	$8*10^3$	$<2.5*10^4$	<250
B. cereus	$2*10^3$	$<2.5*10^4$	<100

For tempeh there does not seem to be any set limits for *Enterobacteriaceae* like there are for meat, dairy, egg, and infant formula according to (EG) nr 2073/2005. It is recommended to test for *Enterobacteriaceae* in the food as unusually high concentrations is a warning bell that something is amiss. This is especially the case if there is a heat treatment step prior to sampling. When that occurs the Swedish Food Agency recommends launching a further investigation by quantifying pathogens like *Salmonella* spp and pathogenic *E. coli* (Livsmedelsverket, 2021). The *Enterobacteriaceae* are likely coming from some form of contamination present in the air of the pilot hall or the incubator itself.

This does not mean that the oat tempeh with pH 5.5 is unsafe as the tempeh is cooked before consumption. The concentration of *B. cereus* is below typical hazardous levels which indicate that it is safe to eat. By lowering the pH to 4.75 there are even lower levels of *B. cereus.*

Bacillus cereus enterotoxin detection

The enterotoxin detection kit is typically used to diagnose *B. cereus* food poisoning, as the toxin itself is very sensitive and gets easily destroyed when heat treated, and thus the concentration of this toxin is of lesser importance. The purpose of the enterotoxin detection was to see if *B. cereus* toxins are indeed present in the food, which indicates that cereulide may also be present. Cereulide cannot be easily removed from the food and is much harder to detect than the enterotoxin. This is the reason that detection of enterotoxin was chosen instead. To detect cereulide more advanced methods like Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry may be used to properly quantify toxin concentration (Dietrich, et al. 2021).

The well plates from the enterotoxin kit showed negative results for all the raw samples of oat, fava, and soy. The concentration of enterotoxin was thus <4ng/ml food extract for the raw material. A photograph of the well plate with the raw samples is shown in Figure 11.

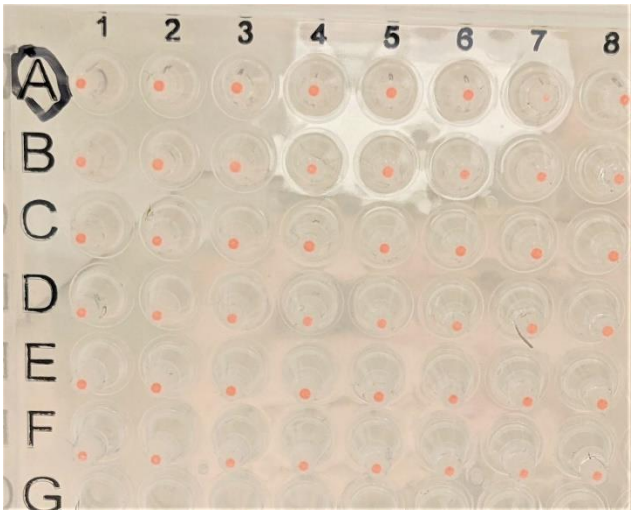

Figure 5: A photograph showing a well plate with results from the enterotoxin kit. Rows A&B are soy okara, C & D are fava bean press cake and E&F is oat press cake.

The results are, like the VRBD plates, not very surprising, as the heat treatment from the decanter should deactivate the sensitive enterotoxin. Subsequent freezing for a prolonged period may also influence the concentrations of enterotoxin.

The enterotoxin detection kit was also used on the finished oat tempeh from trials 6-7. The tempeh from Trial 6 was oat tempeh that was boiled either vacuum sealed or mixed in the plastic bag. The results from this plate show positive results on the first well for both samples, but not subsequent wells. The concentration of enterotoxin is 40 ng/g tempeh after correcting with the dilution factor. A photograph of the well plate with the tempeh samples from trial 6 is shown below in Figure 12.

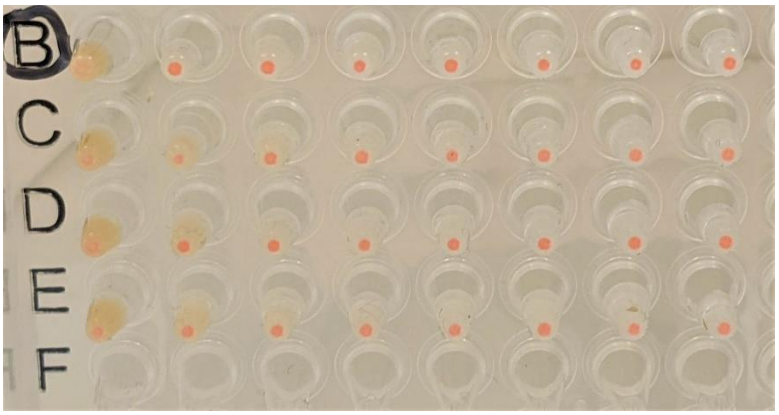

Figure 6: Row B & C are from the tempeh where the oat press cake was mixed during boiling and row D & E are without mixing.

That both samples showed a positive result was surprising since the plate results from trial 6 (Table 18) show no *B. cereus* colonies for either tempeh. One possible explanation could be that the *B. cereus* colonies were present in an early stage of the fermentation, created the toxin and then were outcompeted by the *B. subtilis*.

The tempeh used from trial 7 was oat tempeh with 5.0 and 5.5 pH. The result from this plate is invalid as the 8th well plate shows positive results. This is, according to the manufacturer, a sign that the agglutination reaction is not working properly.

It would have been interesting to do additional trials with the plate count and the enterotoxin kit combined. This could allow a comparison between the *B. cereus* colony count and the enterotoxin concentration in the food.

6 Conclusions

The results demonstrate that is possible to ferment the press cakes made from oats and fava beans into tempeh with *R. oligosporus.* Finished tempeh products made from both fava bean and oats satisfied the evaluation criteria of firmness, visual appearance, mycelia coverage and smell. The press cakes underwent a large transformation in 48 hours, from raw material into firm mycelia-bound tempeh. Finished tempeh smells earthy and mushroom-like, is fully covered in mycelia and has little to no sporulation, or other discoloration. That the tempeh fulfills the evaluation criteria is not, however, a guarantee that the tempeh is safe for consumption. Food safety must be the priority when there are credible microbial risks.

Adjusting the pH with vinegar had an effect in reducing bacterial colony count. The best inhibitory effect while still resulting in tempeh fulfilling the evaluation criteria was achieved at pH 5.0. There the *Enterobacteriaceae* and *Bacillus* spp count was minimised. At pH 5.0, the oat tempeh was well below hazardous levels of *Enterobacteriaceae* and *B. cereus* and could be deemed as safe. Lowering the pH further was undesirable due to inhibition of mycelia growth.

Measuring and monitoring the moisture content may help in both reducing microbial growth and in improving mycelia formation.

The fermentation of the press cakes with LAB was deemed as safe. The *B. cereus* count and *Enterobacteriaceae* colony count was below the detection limit for both yoghurt cultures. There were, however, log 3 *B. subtilis* colonies in oat press cake after fermentation with one of the yoghurt cultures. LAB can reduce bacterial count by bacteriocins and increased acidity.

From the safety investigation, a few conclusions could be drawn. *R. oligosporus* can grow on *Bacillus* ChromoSelect which makes microbial evaluation of the tempeh on this agar difficult as plates were often smeared and difficult to quantify. A different approach should be used for enumeration of *B. cereus*.

7 Future considerations

The next step in the tempeh press cake product development would likely be to conduct sensory evaluations to assess the acceptance of the different tempeh. This could for instance be evaluating taste, texture, aroma and other factors for the oat and fava bean tempeh.

Additionally, further work needs to be done in determining product properties such as protein, fibre, and carbohydrate content. Monitoring of antinutritional factors should also be considered.

To further investigate the effect of temperature and RH on *R. oligosporus* growth during fermentation, enzyme activity or fungal colony count could be analysed. Additionally, a camera taking photos in a timelapse could be set up to visually monitor the mycelia growth at different conditions.

The thesis did not focus on optimizing heat treatment as this can be adjusted in a much more controlled way industrially. For example, a “double pasteurisation” could be used. In this method the food is pasteurised once and then the food is allowed to rest at 30 °C for around 2 hours, allowing the spores to vegetate. The food is then quickly heat treated again. The vegetative cells are vulnerable to this heating method and can reduce *B. cereus* and other microbes to safe levels (Andreasson, Bergwik and Christiansson, 1991).

To increase the selectivity and exclusivity of the plate count there are other agars commercially available that may be more suitable. One of the agars that seems to be a good fit is the CHROMagar™ *B. cereus* (BRILLIANCE *BACILLUS* CEREUS AGAR, 2022). This agar performed better than the *Bacillus* ChromoSelect agar in a study from 2022. There is added Trimethoprim which blocks folic acid synthesis on some non-cereus *Bacillus* species, inhibiting growth (Fuchs et al., 2022). A fungicidal agent could also be used when plating tempeh, as the mould colony growth makes enumeration difficult.

Genomic sequencing could allow identification down to strain level of the bacteria present in the tempeh. This could be used for monitoring *B. cereus* strains, and if toxin producing genes, especially genes coding for cereulide, are present.

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9 Appendix

A. Press cake tempeh batches

Batch 1 lab protocol

Purpose:
First attempt to create tempeh
Material used:
Soy Okara
Fava bean press cake
Oat press cake
Starter culture Njordstorp
Process
Heat treatment
Boiled bags of soy okara, fava bean and oat press cake in pots with around 1 l water in them for 30 minutes. The contents of the bags were put in separate bowls and left to cool.
Inoculation
When the temperature of the press cakes was below 30 °C 3g starter culture /kg press cake was added. Mixed for 2 minutes with a kitchen spoon.
Bag preparation
Nine 0.4 L Ziplock bags were perforated in a grid pattern with a toothpick. The bags were filled with inoculated press cake, around 150 g per bag. The bags were lightly pressed. Oat could fit less weigh inside due to being less dense.
Incubation
The bags were placed in airtight plastic boxes next to each other. Two bags in each box. Inside each box was a bowl of water to increase the humidity slightly. The boxes were then placed inside an incubator at 30 C for 48 hours.
Results
Soy tempeh:
A moist and wet cake, with water droplets on the inside. Some of the surface is covered in mycelia, but most is uncovered. Bottom part of the tempeh not covered by mycelia, except near the edges. Not holding together at all. Shown in figure A1.

Figure A1: Soybean okara tempeh from batch 1.

Fava bean tempeh:
An almost firm homogenous cake that was covered in mycelia on the top was observed. It held when lifted and did not break when gently shaken. The cake did crumble when cut, especially near the bottom part. The bottom side was not covered in mycelia, except near the edges. Shown in figure A2.

Figure A2: Fava bean press cake tempeh from batch 1.

Oat tempeh:
A firm homogenous cake with top covered in mycelia. Bottom part was, like the other cakes, not completely covered. The cake crumbled quite a lot when cut, however. Shown in figure A3.


Figure A3: Oat press cake tempeh from batch 1.

Remarks
Maybe the cake needs aeration on the bottom side. Next batch will try to remedy this by doing a "vertical" incubation.

Batch 2 lab protocol

Purpose
Try letting the inoculated bags hang vertically in the fermentation chamber to allow for free air flow. May improve results (see previous batch).
Material used
Soy okara
Fava bean press cake
Starter culture Njordstorp
Process
Heat treatment
Boiled bags of soy okara and fava bean press cake in pots with around 1 l water in them for 30 minutes. The contents of the bags were put in separate bowls and left to cool.
Inoculation
When the temperature of the press cakes was below 30 C 3g starter culture /kg press cake was added. Mixed for 2 minutes with a kitchen spoon.
Bag preparation
Six 0.4 L Ziplock bags were perforated in a grid pattern with a toothpick. The bags were filled with inoculated press cake, around 150 g per bag. The bags were lightly pressed.
Incubation
A stand with "arms" on which the perforated plastic bags could be hung using tape was constructed. The bags can hang freely in the air, which should allow free airflow to all sides of the bag.

The bags were placed in the airtight plastic boxes hanging vertically on the stand. Four bags in each box. Inside each box was a bowl of water to increase the humidity slightly. The boxes were then placed inside an incubator at 30 C for 48 hours.
Results
Fava bean:

Barely any growth of mycelia. A little bit around some air holes but growing outwards instead of inside the cake! There is no mycelia structure inside the bags. Shown in figure A4.

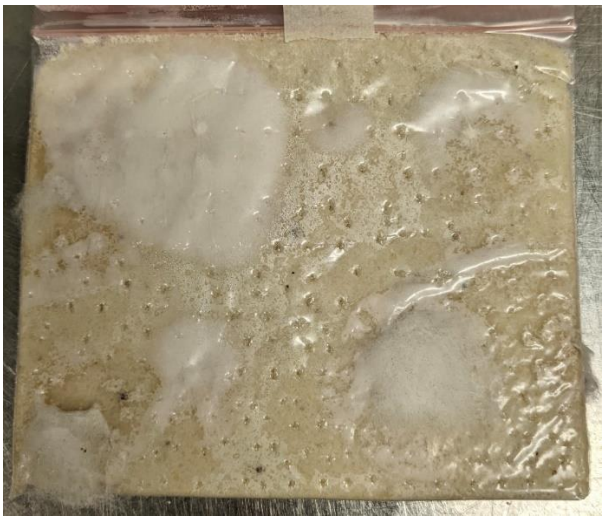

Figure A4: Fava press cake tempeh from batch 2.

Soybean:
Barely any growth of mycelia. Some growing outwards, but just like the fava bean, there is no cohesive structure.

Remarks

It seems that the vertical incubation did not have the intended effect. Maybe the press cake gets pressed into the perforations which prevents the air from entering the Ziplock bags.

Next trial will instead try to make sure that the bags are “airier” and don’t get press cake stuck in the air holes.

Batch 3 lab protocol

Purpose

Another try with the vertical incubation to see if it’s an idea worth anything. This time using soy and oat press cake.

Material

Soy okara
Oat press cake
Starter culture Njordstorp

Process

Heat treatment

Boiled bags of oat and fava bean press cake in pots with around 1 l water in them for 30 minutes. The contents of the bags were put in separate bowls and left to cool.

Inoculation:

When the temperature of the press cakes was below 30 C 3g starter culture /kg press cake was added. Mixed for 2 minutes with a kitchen spoon.

Bag preparation

Six 0.4 L Ziplock bags were perforated in a grid pattern with a toothpick. The bags were filled

with inoculated press cake, around 100 g per bag.

Incubation

The same stands as the last batch were used. The plastic bags can hang freely, and this time the bags were filled with 100g. The bags were placed in the airtight plastic boxes hanging vertically on the stands. Three bags in each box. Inside each box was a bowl of water to increase the humidity slightly. The boxes were then placed inside an incubator at 30 C for 48 hours.

Results

Oat tempeh:

The first time the oat was used. White mycelia is covering all of the surface of the press cake, and the cake itself is firm. Some parts of the "top" broke off and fell to the bottom of the bag. The seems to be a successful fermentation. Shown in figure A5.

Figure A5: Oat press cake tempeh from batch 3.

Soybean tempeh:

The top part of the tempeh is covered with mycelia on both sides. The bottom part got squished and did not grow as well. When this part was cut it crumbled. The top part did not crumble when cut. Shown in figure A6.

Figure A6: Soy press cake tempeh from batch 3.

Remarks
It seems that there is no issue for the tempeh mould to grow at the specified conditions. The way of incubating vertically sounded like a good idea to solve the issue of airflow, but it seems a little bit too fickle, and the cakes themselves fall apart when hanging. I have not found any sources that use this method, so it should be possible to improve airflow in the fermentation chambers instead.

B. Press cake tempeh trials

Trial 1

Purpose
First trial for creation of tempeh. The aim is to use the four evaluation criteria, Firmness, coverage, smell and visual appearance, on the tempeh. This trial uses an online recipe. (https://www.tempehtation.uk/okara-tempeh)
Material used
Soy Okara
Fava bean press cake
Starter culture Njordstorp
Process
Heat treatment
One bag of fava press cake and one bag of soy okara were used. The exact same method was used for both samples.The content was poured into a pot filled with water (one for each bean) and then boiled for 30 minutes. The excess water was drained away using strainers. When **no more** water was dripping from the strainer, the press cake was moved to a frying pan on medium heat under constant stirring for 30 minutes. Afterwards the press cake was left to cool down to room temperature.

Dry samples were collected by filling a 20 ml tube with okara (one each).
Inoculation
15 g Rice flour / kg press cake was added to 3g tempeh starter /kg press cake and mixed. The press cakes were weighed and inoculated. The inoculated material was mixed by hand for 2 minutes with a kitchen spoon.
Bag preparation
Eight 0.4 L Ziplock bags were perforated in a 5 x 5 grid pattern evenly spaced apart with a toothpick (1 mm holes). The bags were filled with inoculated press cake, around 200g per bag. There were four bags each of soy and fava press cake. The bags were lightly pressed to make them more uniform.
Incubation
The bags were placed in airtight plastic boxes next to each other. Two bags in each box. Inside each box was a bowl of water to increase the humidity. Humidity was not controlled during this experiment. The boxes were then placed inside an incubator at 30 C for 48 hours.
Results
Picture from Trial 1 are shown in figure A7 below.

Figure A7: Soy okara tempeh (left) and Fava bean tempeh (right) from trial 1.

Soy tempeh:
White Mycelia covers most of the surface of all the tempeh. Bottom is partly uncovered. Doesn't crumble and can be held without crumbling. Has a nice “clean” smell. Dry matter analysis moisture content 76%, same as raw material.

Fava tempeh:
The fava tempeh was less successful. The mould does not cover most of the cake and it is not spongy. Very wet bags. Does not hold firm, cracks form when held. The moisture content was 82% which is higher than the raw material. Many water droplets on the inside (condensation) of the bags. Maybe more drying is required?

The tempeh according to the evaluation criteria are shown in table A1.

Table A1: Evaluation criteria from trial 1. Firmness, mould coverage, visual appearance and smell are evaluated based on examination of the fermentation results.

Trial 1	Firmness	Mould coverage (%)	Visual appearance	Smell
Soy	Ok	80	White mould	Ok
Soy	Ok	85	White mould	Ok
Soy	Ok	85	White mould	Ok
Fava	Not ok	20	White mould	Ok
Fava	Not ok	10	White mould	Ok
Fava	Not ok	20	White mould	Ok

Remarks
Fava was probably too wet. Was good to use dry matter analysis to get a better picture of what may have gone wrong.

Since soy had the same MC as the raw material and it worked to create tempeh, and the same was true for fava bean press cake, it should be possible to grow tempeh at that MC. This can be done by just boiling inside the bags instead. This was tried for subsequent trials.

Trial 2

Purpose:
Second trial of product development of tempeh. This trial I tried mixing the press cakes;
75/25 Fava/Oat and 50/50 Fava/Oat.
Unmixed fava bean and oat tempeh was also created.
The aim is to use the four evaluation criteria, Firmness, coverage, smell and visual appearance, on the tempeh.

Material used:
Fava bean press cake
Oat press cake
Starter culture Njordstorp

Process
Two bags of fava press cake and one bag of oat press cake were used. The exact same method was used for both samples.

Heat treatment
The vacuum sealed bags of fava bean and oat press cake were put into pots filled with water and boiled for 30 minutes.
Afterwards the press cake was left to cool down to room temperature in separate bowls.

Dry samples were collected by filling a 20ml tube with press cake.

Inoculation

15 g Rice flour / kg presscake was added to 3g tempeh starter /kg presscake and mixed. The press cakes were weighed and inoculated. The inoculated material was mixed by hand for 2 minutes with a kitchen spoon.

Two separate bowls with 50/50 fava/oat and 75/25 fava/oat with approx. 450 g in each were weighed up.

Bag preparation

10 0.4 L Ziplock bags were perforated in a 5 x 5 grid pattern evenly spaced apart with a toothpick (1 mm holes). The bags were filled with inoculated press cake, around 200g per bag. There were three bags of fava bean, three of oat. Two bags each of 75/25 Fava/Oat and 50/50 Fava/Oat were filled.

The bags were lightly pressed to make them more uniform.

Incubation

The bags were placed in airtight plastic boxes next to each other. Two bags in each box. Inside each box was a bowl of water to increase the humidity. Humidity was not controlled during this experiment. The boxes were then placed inside an incubator at 30 C for 48 hours.

Results

Oat tempeh:

Completely failed. Strong sewer smell coming from the bags. Very wet. Moisture leaking out from holes. Barely any mycelia. 73% Moisture content was apparently too much. Shown in figure A8.

Figure A8: Oat tempeh from trial 2.

Fava tempeh:

Very cracked surface for some reason. White mycelia is binding the “cracks” in the surface holding it together. Not very stable and breaks easily when held. Not crumbly when cut. Worrying red spots on surface!!! Smells a little like ammonia, may be overripe. 77 % MC. Shown in A9.

Figure A9: Fava bean tempeh from trial 2.

50/50 Fava/Oat:
Very cracked surface, but pretty good mycelia coverage. Hard to judge what the percentage is though based on the cracks. Pretty good coverage of most of the underside (except the bottom part of the bag. Holds up when lifted for a short while. Is firm. Smells like ammonia. Shown in A10.

Figure A10: Fava bean/Oat 50/50 tempeh from trial 2.

75/25 Fava/Oat:
Very cracked surface, but pretty good mycelia coverage. Hard to judge what the percentage is though based on the cracks. Holds up when lifted for a short while, but the cracks increase in size until failure. Product has a strong ammonia-like smell. Shown in figure A11.

Figure A11: Fava bean/Oat 75/25 tempeh from trial 2.

The tempeh according to the evaluation criteria are shown in table A2.

Table A2: Evaluation criteria from trial 2. Firmness, mould coverage, visual appearance and smell are evaluated based on examination of the fermentation results.

Trial 2	Firmness	Mould coverage (%)	Visual appearance	Smell
Oat Tempeh	No	0	No	Sewer smell!
Oat Tempeh	No	0	No	Sewer smell!
Oat Tempeh	No	0	No	Sewer smell!
Fava Tempeh	No	60	White mould	Ammonia, ok
Fava Tempeh	No	60	White mould	Ammonia, ok
Fava Tempeh	No	65	White mould	Ammonia, ok
50/50 Fava/Oat	Yes	90	White mould	Ammonia, not ok
50/50 Fava/Oat	Yes	90	White mould	Ammonia, not ok
75/25 Fava/Oat	Almost	75	White mould	Ammonia, not ok
75/25 Fava/Oat	Almost	75	White mould	Ammonia, not ok

Remarks:
Something went seriously wrong with Oat tempeh. I wonder if it was contaminated, or that it was too moist. Very worrying smell. Still MC is lower than for fava beans, but it leaked water. Does fava bean have a higher moisture holding capacity? Can this be measured or seen in literature?

The fava tempeh was better than the oat tempeh, but still something was off about this batch. The cracks formed may have something to do with how the inoculated press cake is pressed in the bags to make them homogenous.

After two days of refrigeration, the fava tempeh was growing red spots! Through an error search and conferring with microbiological experts, the cause was most probably the rice flour. Future trials will not use rice flour as it is typically highly contaminated.

Trial 3

Purpose
Third trial of product development of tempeh. This trial used fava oat and soy. The purpose was to perform the same steps as Trial 2 and see if I can find out what went wrong in the fermentation. Why there were “cracks” and if rice flour created the red spots after refrigeration were investigated.
Material
Fava bean press cake
Soy okara
Oat press cake
Starter culture Humlegårdens ekolager.
Process
One bag of each press cake was used. For this trial humlegårdens tempeh starter was used as the one from Njordstorpsgård ran out and the replacement culture had not yet arrived.
Heat treatment
The vacuum sealed bags of fava bean, oat press cake and soybean okara were put into pots filled with water and boiled for 30 minutes.

Obs!! Here I noticed that the bag containing oat press cake had small holes in it! This led to water entering the “vacuum sealed” bag and thus increasing the MC.

Afterwards the press cake was left to cool down to room temperature in separate bowls.
The dry samples were collected by filling a 20ml tube with press cake.
Inoculation
The press cakes were weighed and inoculated with 3g tempeh starter /kg press cake and mixed.
The inoculated material was mixed by hand for 2 minutes with a kitchen spoon.
Bag preparation
9 0.4 L Ziplock bags were perforated in a 5 x 5 grid pattern evenly spaced apart with a toothpick (1 mm holes). The bags were filled with inoculated press cake, around 200g per bag. There were two bags of fava bean, and oat press cake. Three bags were filled with soy okara. The bags were lightly pressed to make them more uniform.
Incubation
The bags were placed in airtight plastic boxes next to each other. Two bags in each box. Inside each box was a bowl of water to increase the humidity. Humidity was not controlled during this experiment.

The boxes were then placed inside an incubator at 30 C for 48 hours.
The progress was checked after 24 h of fermentation.

Results
After 24 h:
Soy tempeh has a lot of white mycelia on top! Solid cake. Almost covering the entire surface of the tempeh!

Fava has a little white on top. Barely visible but is mostly uncovered. Cake feels a bit firmer than it was before fermentation but is not solid.

Oat feels firm and has some white mycelium on it, especially around the edges.
After 48 h:

Soy tempeh:
Cake is not firm. Surfaces are mostly covered but the inside is not. Mould has not penetrated the inside. The surface is a little wet to the touch. Doesn't smell bad though. Brown spots in one of the soy tempeh. Shown in A12.

Figure A12: Soy tempeh from trial 3.

Fava bean tempeh:
Solid firm cake. Completely covered on the surface but seems to be plenty of mycelia on the inside. Not wet or slimy surface. Tastes funghi like. Can be cut into pieces and fried without crumbling. The texture was great, like fried tofu but chewier. It tasted mushroomy with a hint of fava bean. Shown in A13.

Figure A13: Fried fava tempeh from trial 3.

Oat tempeh:
Very solid cake and excellent mould growth. This seems to have gone very well. Crumbles a little bit when cut. When fried it gets a crispy surface. This is maybe because of the high dry matter content. Smells good too. Shown in A14.

Figure A14: Oat tempeh from trial 3.

The tempeh according to the evaluation criteria are shown in table A3.

Table A3: Evaluation criteria from trial 2. Firmness, mould coverage, visual appearance and smell are evaluated based on examination of the fermentation results.

Trial 3	Firmness	Mould coverage (%)	Visual appearance	Smell
Oat Tempeh	Yes	95	White mould	Ok
Oat Tempeh	Yes	95	White mould	Ok
Fava Tempeh	Yes	100	White mould	Ok
Fava Tempeh	Yes	100	White mould	Ok
Soy tempeh	No	45	Brown spot	Ok
Soy tempeh	No	55	White mould	Ok
Soy tempeh	Yes	55	White mould	Ok

Remarks:
Strange that the soy tempeh seems to be doing bad after the full fermentation. After 24h it looked like it was doing the best of the three.
No cracks appeared like in trial 2. It is unclear what I did to make that happen. If it occurs again,

a more thorough investigation will be conducted.
It seems that after refrigeration no red spots appeared on the fava or oat tempeh. (The soy tempeh was discarded before refrigeration). It was probably the rice flour that was contaminated.

Trial 3.5

Purpose
Third (.5) trial of product development of tempeh. This trial used fava beans. I wanted to have samples for The Green Dairy.
Material
Fava bean press cake
Starter culture Humlegårdens ekolager.
Process
One bag of fava was used. For this trial humlegårdens tempeh starter was used as the one from Njordstorpsgård ran out and the replacement culture had not yet arrived.
Heat treatment
The vacuum sealed bag of fava bean press cake was put into a pot filled with water and boiled for 30 minutes. Afterwards the press cake was left to cool down to room temperature in separate bowls.

The dry samples were collected by filling a 20ml tube with press cake.
Inoculation
The press cakes were weighed and inoculated with 3g tempeh starter /kg presscake and mixed. The inoculated material was mixed by hand for 2 minutes with a kitchen spoon.
Bag preparation
2 1.2 L Ziplock bags were perforated in a 5 x 5 grid pattern evenly spaced apart with a toothpick (1 mm holes). The bags were filled with inoculated press cake, around 400g per bag. The bags were lightly pressed to make them more uniform.
Incubation
The bags were placed in airtight plastic boxes next to each other. Two bags in the box. Inside each box was a bowl of water to increase the humidity. Humidity was not controlled during this experiment.
The boxes were then placed inside an incubator at 30 C for 48 hours.
Results:
There was no growth at all of mycelia on the surface surprisingly. It looks like the growth may have started, as it smells like tempeh (and also the smell of raw fava bean press cake remains). The cake itself is very spongy, but it is not very firm. It crumbles when lifted. Holds the shape of the bag a little bit though so there seems to have been some growth.
When the box was opened the air felt very moist.
The tempeh according to the evaluation criteria are shown in table A4.

Table A4: Evaluation criteria from trial 3.5. Firmness, mould coverage, visual appearance and smell are evaluated based on examination of the fermentation results.

Trial 3.5	Firmness	Mould coverage (%)	Visual appearance	Smell
Fava bean	No	0	Spongy, no mould	Ok
Fava bean	No	0	Spongy, no mould	Ok

Remarks:
MC is 72% so it shouldn't be an issue with MC as previous batches have had even higher and succeeded for the fava bean.
Incubation itself: By checking common sources of error for tempeh production the most likely cause may be that the mycelia died of heat. When the *Rhizopus oligosporus* grows it generates heat. Maybe having 800 g total of fava in one fermentation chamber is too much.
The idea of the fermentation chamber seems limiting. Though it offers an easier way to make sure the fermentation area is sanitised, it also is limited in airflow, temperature control (no air exchange may increase temperature) and humidity.

It could also be the starter culture itself that is not as potent as it should be.

Trial 4

Purpose
Fourth trial of product development of tempeh. This trial used fava and oat press cake. Two types of fava press cake were used, batch from 2022-01-27 and 2021-02-19. The batch from 2021 was running out. The fava press cakes look very different.

The aim was to use the blast freezer incubator instead of the fermentation chambers. The freezer has a 90 C setting which can kill mould after usage.

Material used
Fava bean press cake 2022-01-27 and 2021-02-19
Oat press cake
Starter culture Humlegårdens ekolager.

Process
One bag of each press cake was used. For this trial humlegårdens tempeh starter was used as the one from Njordstorpsgård ran out and the replacement culture had not yet arrived.

Heat treatment
The vacuum sealed bags of fava bean and oat press cake were controlled to see if they had any holes or damages on them. If they did, the "vacuum" bag was placed inside a new and undamaged vacuum bag.
Then the bags were placed in pots filled with water and boiled for 30 minutes. The timer was started when the water was at 100 C.

Afterwards the press cake was left to cool down to room temperature in separate bowls.

The dry samples were collected by filling a 20ml tube with press cake.

Comparison between the press cakes shown in figure A15.

Figure A15: Comparison of the raw materials. Oat 2019 (left), Fava bean 2021 (centre) and fava bean 2022 (right).

Very different colours on fava bean batches! Fava bean 2022 is more wet and has more clumps. The colour is very off. It is unclear what the reason for this is. The Green Dairy did not specify.

Inoculation

The press cakes were weighed and inoculated with 3g tempeh starter /kg presscake and mixed. The inoculated material was mixed by hand for 2 minutes.

Bag preparation

9 0.4 L Ziplock bags were perforated in a 5 x 5 grid pattern evenly spaced apart with a toothpick (1 mm holes). The bags were filled with inoculated press cake, 150-200g per bag (fava 2019 & oat 2019 150g, fava 2022 200g. There were two bags of fava bean (one from each batch), and oat press cake. Three bags of each kind of press cake. The bags were lightly pressed to make them more uniform.

Incubation

The incubator was set to 30 C and 70% RH for 48 hours.
The bags with press cake were put into the incubator evenly spaced around.
A Temperature & RH logger was placed inside the incubator.

The progress was checked after 24 h of fermentation.

Results

24h fermentation report:
The oat tempeh has formed very nice firm cakes covered in mycelia. Beautiful cakes. Smells really good too.

Fava bean2021 has some growth of mycelia near air holes but isn't very firm. Still very wet, water droplets forming on the inside of the bags.

Fava bean 2022* (wrong date on label on tempeh)
Has a thin layer of mycelia on top and are firm cakes. Smells good too!
48h ferment report:
Oat tempeh was fully covered, the bags filled up. No signs of sporulation. Looks beautiful. Very good smell of mould. Firm, dry cake. Bag was filled up with mycelia. Doesn’t crumble, tastes

good when cooked too, kind of like mushrooms. Very fibrous though. The texture really does change quite a lot after cooking. Good smell and no discoloration. Shown in A16.

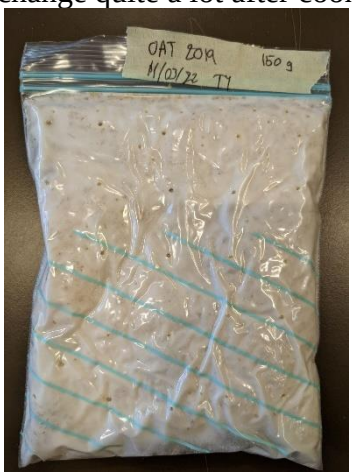

Figure A16: Oat tempeh from trial 4.

Fava 2021 tempeh was fully covered, and the entire bag was full. No sporulation and good firmness and doesn't crumble. Good smell and no discoloration. Shown in A17.

Figure A17: Fava bean 2021 tempeh from trial 4.

Fava2022 Gloria was partially covered in white mycelia. No off smell, so I don't think it's contaminated. Probably high moisture content that made the fermentation go sub optimally. Shown A18.

Figure A18: Fava bean 2022 tempeh from trial 4.

The results from the criteria evaluation are shown in the table A5 below.

Table A5: Evaluation criteria from trial 4. Firmness, mould coverage, visual appearance and smell are evaluated based on examination of the fermentation results.

Trial 4	Firmness	Mould coverage (%)	Visual appearance	Smell
Fava Bean 2022	No	40	White mycelia	Ok
Fava Bean 2022	No	35	White mycelia	Ok
Fava Bean 2022	No	40	White mycelia	Ok
Fava Bean 2021	Yes	100	White mycelia	Ok
Fava Bean 2021	Yes	100	White mycelia	Ok
Fava bean 2021	Yes	100	White mycelia	Ok
Oat 2019	Yes	100	White mycelia	Ok
Oat 2019	Yes	100	White mycelia	Ok
Oat 2019	Yes	100	White mycelia	Ok

Remarks
Using the freezer incubator was great. The oat 2019 and fava 2019 tempeh were great! Nice firm cakes of good quality. The oat okara had beautiful growth after just 24 hours. The fav hadn't grown so much after 24, but 48h in was looking very good.

The fermentation chamber does not create this sort of results. In a closed environment the spores may die of overheating. The incubator regulates the temperature and humidity. With the logger data, it was easy to see that the temperature stays steady for most of the cultivation time, with no huge jumps though the data jumps a bit.

I will try drying the fava Gloria in an oven so the MC is lowered, which may improve fermentation**.**

Trial 5

Purpose

Fifth trial of product development of tempeh. This trial used fava 2022 and oat press cake. The fava 2021 ran out, and there is no more presscake from this batch. Will have to make do with the fava 2022 even though it seems to be lower quality.
The aim of this trial was to use the freezer incubator again to see if the same good results for the oat tempeh could be replicated. The fava 2022 was dried in an attempt to lower the MC. The same incubator settings were used as trial 4.

Now the tempeh starter from Njordstorpsgård arrived and will be used instead of the tempeh purchased from humlegårdens ekolager.

Samples were taken from the finished tempeh and plated.

Material used

Fava bean press cake 2022-01-27
Oat press cake 2019
Starter culture Njordstorpsgard

Process

Heat treatment

The vacuum sealed bags of fava bean and oat press cake were controlled to see if they had any holes or damages on them. If they did, the “vacuum” bag was placed inside a new and undamaged vacuum bag. Then the bags were placed in pots filled with water and boiled for 30 minutes. The timer was started when the water was at 100 C°.

The fava presscake was spread out on an oven plate and put in an oven at 60C° for 1 hour. A lot of water evaporated, and there was only enough press cake to fill two bags.
Afterwards the press cake was left to cool down to room temperature in separate bowls.
The dry samples were collected by filling a 20ml tube with press cake.

Inoculation

The press cakes were weighed and inoculated with 3g tempeh starter /kg presscake and mixed.
The inoculated material was mixed by hand for 2 minutes.

Bag preparation

5 0.4 L Ziplock bags were perforated in a 5 x 5 grid pattern evenly spaced apart with a toothpick (1 mm holes). The bags were filled with inoculated press cake, 150-200g per bag (oat 2019 150g, fava 2022 200g). Three bags were filled with oat press cake and two bags of fava. The bags were lightly pressed to make them more uniform.

Incubation

The incubator was set to 30 C and 70% RH for 48 hours.
The bags with press cake were put into the incubator evenly spaced around.
The progress was checked after 24 h of fermentation.

Results

24 h fermentation:

Oat 2019 tempeh cakes were firm and had a little of mycelia on top of them.
Fava 2022 tempeh cakes had quite a lot of white mycelia. Seemed firm.

48 h fermentation:

Oat 2019

Oat cakes are firm and fully covered in mycelia. Some sporulation has occurred but in limited areas.
When cutting with a knife it is a little crumbly and eating it raw has a fairly dry mouthfeel. Frying in oil for 4 minutes (with stirring) makes the outside crispy and the inside feel moister. The texture changes. Keeps their shape and doesn't disintegrate when fried. Tasted mushroomy.

Fava 2022

The Fava 2022 tempeh cakes were a bit slimy on the outside. Dark, grey and doesn't look very attractive. The tempeh has a strong smell of ripened cheese. The smell is very concerning and indicative of something growing there that shouldn't. The mycelia doesn't cover the surface anymore. *R. oligosporus* maybe got outcompeted. The cake is a little bit crumbly when cut but holds up well when lifted.

The results from the criteria evaluation are shown in the table A5 below.

Table A5: Evaluation criteria from trial 5. Firmness, mould coverage, visual appearance and smell are evaluated based on examination of the fermentation results.

Trial 5	Firmness	Mould coverage (%)	Visual appearance	Smell
Fava Bean 2022	Yes	~5	Dark, slimy	No
Fava Bean 2022	Yes	~5	Dark, slimy	No
Oat 2019	Yes	100	Minor black sporulation	Ok
Oat 2019	Yes	100	Minor black sporulation	Ok
Oat 2019	Yes	100	Minor black sporulation	Ok

Remarks

To find out what happened to the fava 2022, it was plated on VRBD and *Bacillus* ChromoSelect.
The oat tempeh has shown to create tempeh cakes of good quality in two trials. It would be interesting to know how the microbiota looks by plating.

The incubation strategy clearly works. Will try the original lab incubator again and monitor conditions using a data logger to see if the humidity matters.

Trial 6

Purpose

The sixth trial of tempeh product development. The purpose is to check if there is any noticeable difference in growth when using the lab incubator compared to trial 5. Only oat press cakes will

be tried. It seems that the fava 2022 batch has some issue with it. Will request more fava bean press cake.
Different boiling methods will be used and then check with the microbial concentration by aerobic plate count. Checking if mixing the presscake will have a difference. Oat presscake with pH adjusted to 4.5 (overkill) will also be tried.

Material used

Oat press cake 2019
White vinegar (6%)
Njordstorp starter culture

Process

Heat treatment

Two bags of vacuum frozen oat press cake were used. One of the vacuum bags was placed directly in the boiling water, the other was opened and put into a larger vacuum bag. This allowed the content to be mixed.
Then the bags were placed in pots filled with water and boiled. Since the plating of tempeh from trial 5 showed a high microbial content, the boiling time was increased to 1.5 hours. The timer was started when the water was at 100 C°. Every 20 minutes the contents were mixed. The temperature of the contents was recorded using an IR- thermometer. Table with temperatures measured A6.

Table A6: Internal temperature for the vacuum bag, measured with an IR thermometer.

20 min	75 C
40 min	85 C
60 min	85 C
80 min	87 C
90 min	86 C

Afterwards the press cake was left to cool down to room temperature in separate bowls.

Inoculation

The amount of vinegar required to lower the pH to 4.5 was calculated to 1.5 ml vinegar/ 10 g oat press cake.
The dry samples were collected by filling a 20ml tube with press cake after adding vinegar!
The press cakes were weighed and inoculated with 3g tempeh starter /kg presscake and mixed.
The inoculated material was mixed by hand for 2 minutes.

Bag preparation

8 0.4 L Ziplock bags were perforated in a 6 x 6 grid pattern evenly spaced apart with a toothpick (1 mm holes). The bags were filled with inoculated press cake, 150 g per bag.
Two bags were filled from each boiling method and 2 bags with vinegar for each boiling method. In total 8 bags were used. The bags were lightly pressed to make them more uniform.

Incubation

The lab incubator was set to 30 C. The bags were placed inside the incubator evenly spaced for 48 h. A temperature & humidity logger was placed inside the incubator.

Results

48 h fermentation:
Bags with vinegar had no visible mycelia except in some corners. Shown in A19.

Figure A19: Oat tempeh with vinegar added from trial 6.

Oat tempeh with no added vinegar have thick mycelium growth. Look visually the same as each other. Firm cakes are fully covered in mycelia, but they have a few spots with black sporulation. They smell good too. Shown in A20.

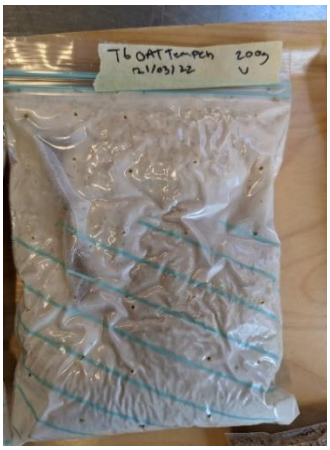

Figure A20: Oat tempeh from trial 6, unmixed boiling method.

The results from the criteria evaluation are shown in the table A7 below.

Table A7: Evaluation criteria from trial 6. Firmness, mould coverage, visual appearance and smell are evaluated based on examination of the fermentation results.

Trial 6	Firmness	Mould coverage (%)	Visual appearance	Smell
Oat mixed	Yes	100	Minor black sporulation	Ok
Oat mixed	Yes	100	Minor black sporulation	Ok
Oat	Yes	100	Minor black sporulation	Ok
Oat	Yes	100	Minor black sporulation	Ok
Oat vinegar mixed	No	0	N/A	Vinegar
Oat vinegar mixed	No	0	N/A	Vinegar
Oat vinegar	No	0	N/A	Vinegar
Oat vinegar	No	0	N/A	Vinegar

Remarks
The pH needs to be higher because mycelia did not grow at pH 4.5. The mixed boiling method works better microbially, probably due to better heat transfer and distribution. The low pH did greatly affect the plate results, so the next trial will be to try a range of pH and see what performs the best, both as a product and bacterial safety.

Trial 7

Purpose
The seventh trial of tempeh product development. The purpose is to check microbial growth for different pH values. The pH tested are 4.75, 5, 5.5, with triplicates. The lab incubator was used since there does not seem to be a noticeable difference in growth even in the lower humidity. Only oat press cakes will be tried.

Material
Oat press cake 2019
White vinegar (6%)
Njordstorp starter culture

Process

Heat treatment
One bag of vacuum frozen oat press cake was used. The contents were transferred to a larger plastic bag that allowed for mixing. Then the bag was boiled in water for 1.5 hours. The timer was started when the water was at 100 C°. Every 20 minutes the content of the bag was mixed. The temperature of the contents was recorded using an IR- thermometer. Table with temperatures measured A8.

Table A8: Internal temperature for the vacuum bag, measured with an IR thermometer.

20 min	71 C
40 min	85 C
60 min	85 C
80 min	89 C

Afterwards the press cake was left to cool down to room temperature in separate bowls.
The dry samples were collected by filling a 20ml tube with press cake.

Inoculation

The amount of vinegar required to lower the pH to 4.75, 5 and 5,5 was determined through experimentation to 1/0.5/0.3 ml vinegar/ 10 g oat press cake.
The press cakes were weighed, the appropriate volume vinegar was added and mixed. The presscake was then inoculated with 3g tempeh starter /kg presscake and mixed. The inoculated material was mixed by hand for 2 minutes.

Bag preparation

9 0.4 L Ziplock bags were perforated in a 6 x 6 grid pattern evenly spaced apart with a toothpick (1 mm holes). The bags were filled with inoculated press cake, 150 g per bag.
Triplicate per pH value. The bags were lightly pressed to make them more uniform.

Incubation

The lab incubator was set to 30 C. The bags were placed inside the incubator evenly spaced for 48 h.

Results

24h fermentation:

Clear mycelia growth on the bags with pH 5.5. The other bags have not yet shown any growth.

After 48 h:

Nice firm cakes from the pH 5.0 and 5.5. Completely covered in mycelia, they smell good and have a good visual appearance. They crumble a little bit when cut but hold firm when lifted. Bags with pH 4.75 smell like vinegar and do not have much observable growth. Mycelia growth is limited to small spots neat edges of the “cake”. Shown in A21.

Figure A21: Oat tempeh from trial 7, pH 4.75.

Tempeh with pH 5.0 formed good looking & smelling cakes that hold firm very well. Shown in A22.

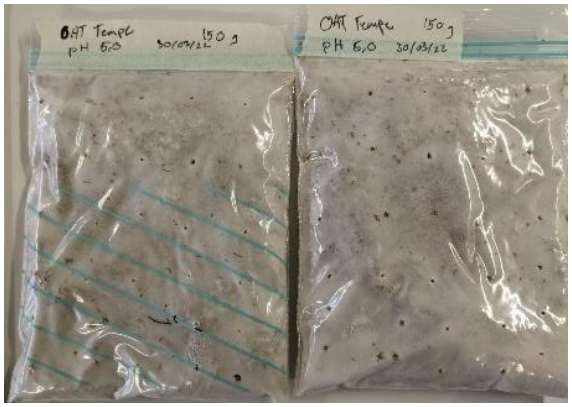

Figure A22: Oat tempeh from trial 7, pH 5.0.

Two of the bags with Tempeh with 5.5 pH have sporulated a little bit near the top of the ziplock bags, but otherwise look perfect. Shown in A23.

Figure A22: Oat tempeh from trial 7, pH 5.5.

The results from the criteria evaluation are shown in the table A9 below.

Table A9: Evaluation criteria from trial 6. Firmness, mould coverage, visual appearance and smell are evaluated based on examination of the fermentation results.

Type	Firmness	Mould coverage (%)	Visual appearance	Smell
Oat pH 4.75	No	0	-	Vinegar
Oat pH 4.75	No	0	-	Vinegar
Oat pH 4.75	No	0	-	Vinegar
Oat pH 5.0	Yes	100	White mycelia	Ok
Oat pH 5.0	Yes	100	White mycelia	Ok
Oat pH 5.0	Yes	100	White mycelia	Ok
Oat pH 5.5	Yes	100	White mycelia	Ok
Oat pH 5.5	Yes	100	Minor black sporulation	Ok
Oat pH 5.5	Yes	100	Minor black sporulation	Ok

Remarks

pH 5 produces a tempeh with a high safety and quality. It seems to be the best regarding the safety (well apart from pH 4.75), but that it seems to take longer time to ferment may be an issue from a cost perspective. I'm pretty happy with the results from the Oat tempeh. I can produce a consistent quality with the method I'm using now. Now to scale up the process might be a good idea.