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Licenciado em Química Aplicada

Grass pea miso: Development of miso based on a portuguese legume - microbiota and preservation capacity

Dissertação para obtenção do Grau de Mestre
em Ciências Gastronómicas

Orientador: Prof. Doutora Catarina Prista, Professora Auxiliar, ISA/UL
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FACULDADE DE CIÊNCIAS E TECNOLOGIA
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2018

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“Many are stubborn in pursuit of the path they chosen, few in pursuit of the goal.”

Friedrich Nietzsche

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Abstract

Fermented foods are extremely popular nowadays mainly thanks to their health benefits and diversity. One example of a fermented food is miso, a fermented soybean paste very famous in Japan, also considered a functional food and used to make soups or as a seasoning for several dishes.

In Japan, miso is made by traditional backslope methods. With the arrival of miso to Western countries, more standard, controlled, sustainable and safe processes are needed, starting by switching the soybean by Portuguese legumes (e.g. grass pea) and by developing starters. The aim of this project is to develop an innovative, tastier and safe grass pea miso, using a yeast starter culture and soybean miso as control.

The characteristics and evolution of miso were evaluated, based on color and microbiota studies. Results obtained point to an important role of *Aspergillus oryzae* at the beginning of fermentation, and to the maintenance of the viability of yeasts (*Candida versatilis*) used as a starter along the whole process of miso's maturation. Also, the obtained product presented good sensorial characteristics being largely accepted by consumers.

The self-preservation capacity of grass pea miso was also evaluated in order to ensure that the product is safe when stored at the consumer's house and shop shelves. Using challenge tests at several temperatures (4ºC, 25ºC and 37ºC), it was observed a substantial reduction in the pathogenic microorganisms' population, in most cases leading to its complete elimination. At the temperatures of 37ºC and 25ºC, all the microorganisms were eliminated (except for *Bacillus cereus)* after 2 days while at 4ºC it takes more than 30 days for them to be eliminated.

Keywords: Fermented foods, miso, grass pea, microbiota, preservation capacity, challenge tests.

Resumo

Os alimentos fermentados têm-se tornado muito populares em períodos recentes muito devido à sua diversidade e aos benefícios que estes trazem à nossa saúde. Um exemplo é o miso, que é uma pasta fermentada de grãos de soja, também considerada uma comida funcional, que é usado para fazer sopa ou como condimento em variadas receitas.

No Japão, existe uma receita tradicional feita há muitos séculos. Com a chegada deste alimento a outros países, métodos mais padronizados, controlados, seguros e sustentáveis são necessários, começando pela substituição a soja por leguminosas portuguesas (como o chícharo) e pelo desenvolvimento de culturas "*starter*". O objetivo deste projeto é desenvolver um miso de chícharo, inovativo, saboroso e seguro para consumo, usando uma cultura "*starter*" de leveduras e o miso de soja como controlo.

As características e evolução do miso foram avaliados, baseado em estudos de cor e de microbiota. Os resultados obtidos apontam para um papel importante de *Aspergillus oryzae* no início da fermentação, e para a manutenção da viabilidade da levedura (*Candida versatilis*) durante o processo de maturação do miso. O produto final apresenta boas características sensoriais sendo muito aceite pelos consumidores.

A capacidade de auto-preservação do miso de chícharo foi também avaliada para que nos assegurássemos que o produto era seguro para ser guardado em casa dos consumidores ou nas prateleiras das lojas. Usando *challenge tests* a várias temperaturas (4ºC, 25ºC e 37ºC) foi observado uma redução significativa da população dos microrganismos patogénicos, em alguns casos levando à sua completa eliminação. Às temperaturas de 25ºC e 37ºC todos os microrganismos patogénicos foram eliminados (excepto *Bacillus cereus*) após 2 dias, enquanto que à temperatura de 4ºC, os microrganismos patogénicos apenas foram eliminados passados 30 dias.

Palavras-chave: Alimentos fermentados, miso, chícharo, microbiota, capacidade preservação, *challenge tests*.

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List of abbreviations

ATM	Atmosphere (Atmosfera)
BLAST	Basic Local Alignment Search Tool
CFU	Colony-forming unit (Unidade formadora de colónias)
DNA	Deoxyribonucleic acid (Ácido desoxirribonucleico)
MRS	De Man, Rogosa and Sharpe agar
NaCl	Sodium chloride (Cloreto de sódio)
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid (Ácido ribonucleico)
RPM	Rotations per minute (Rotações por minuto)
TSB	Tryptic soy Broth
TSA	Trypticase soy agar
UV	Ultraviolet (ultravioleta)
V	Volts
YPD	Yeast extract peptone dextrose

1. Introduction

1

In the present day, people are starting to care more about their health. A lot of people are starting to see that food allergies and intolerances are getting more common and thanks to that they tend to search for alternative healthy food. Fermented foods are usually sought because of their health benefits. These types of foods are very common in Asian countries and only recently started to “arrive” to Western countries (Chilton, Burton, & Reid, 2015).

One example of those fermented foods is miso. Miso is a traditional fermented soybean paste made with mould rice, water, salt and soybeans. Most of miso pastes have a long fermentation time (up to three years) but there are also some types of miso that ferment for few months. Miso has been very popular in Japan for centuries and only now the rest of the world is starting to realize how beneficial this food is. In Japan it is believed that the miso is associated with the longevity of their people (Kailasapathy & Tamang, 2010).

With the growing interest of studying and consuming miso, people are starting to study and develop new ways of making this traditional fermented food. The main ingredient that can be replaced is soybeans and researchers try to replace by more familiar legumes used in their own countries. For this work it was used grass pea instead of the soybean.

The grass pea (*Lathyrus sativus L*.) is a legume which is very rich in proteins, carbohydrates and minerals and resembles the shape of lupin beans although it has a lower protein and higher carbohydrate content (Campbell, 1997). In Portugal, grass pea is part of the traditional heritage of dryland communities, representing an important source of revenue for some local economies. However, there has been a great reduction in consumption of this legume over the years as a result of the lack of innovation on its food products, the emergence of new consumer habits, and an overall unattractiveness of current grass pea-based foods.

The present project integrates a broader FCT funded project (PTDC/AGR-TEC/0992/2014, Deciphering the grass pea (*Lathyrus sativus*) quality riddle. How can the omics technologies contribute to a demand-driven improvement in legume quality?), aiming to increase grass pea consumption and cultivation, by aligning breeding objectives and consumer preferences through the optimization of production and the creation of innovative, attractive, convenient and delicious grass pea-based products.

The main goal of the present work was to evaluate how viable the grass pea is as a replacement to soybeans in making a tasty miso paste and how viable is the production of this miso using a yeast starter composed by *Candida versatilis* and *Zygosaccharomyces rouxii*. More specifically, we evaluated the behavior of grass pea miso inoculated with each of the yeasts and with a mixture of both yeasts in what concerns:

- The evolution of fermentation through color observation (visual observation and CIElab measurements);
- The evolution of miso microbiota (both by standard and molecular methods), using soya bean miso inoculated with the same starters and traditional made grass pea and soya bean miso as control.
- The determination of the shelf-life period and preservation capacity of miso paste made from grass pea was performed by challenge tests with the main food contaminant microorganisms.

2. Literature Review

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2.1. Fermented foods

2.1.1. Historical framework

If we look back in history, fermented foods were most likely the first type of food consumed by human beings. This happened not because early humans had planned on producing and/or eating it, but rather because fermentation was the inevitable outcome of leaving raw food materials in an unpreserved state. For example, the juice of grapes or other fruits would remain sweet for a couple of days before being transformed into a pleasant but intoxicating wine-like drink (Hutkins, 2006).

Fermented foods were not only appreciated because of the sustenance they provided but also for aesthetic and organoleptic reasons. Even after the "discovery" that fermented foods tasted good and were well preserved, it took many years for humans to understand what really happened with them and how to control or influence the conditions to consistently produce more products (Hutkins, 2006; Ornelas & Kiple, 2000).

As long ago as 3000 to 4000 B.C.E, bread and beer were already being mass produced by Egyptian bakeries and Babylonian breweries. It's clear also, through historical records, that the consumption of beer, wine and other fermented products coincided with the rise of civilizations around the Mediterranean, east Europe and the middle East. Beer and wine started being very popular mainly because water was very polluted with fecal material or other materials (Hutkins, 2006; Ornelas & Kiple, 2000).

With the rise and spreading of the Roman Empire, fermentation started being widespread among the whole Empire because they were adopting the raw materials and technologies of the lands they conquered. Fermented foods also had an important role in ships thanks to their increased storage capability. During this era, the means to conduct trading had evolved and people started trading cheese, wine and wheat (to make bread), specially around the Mediterranean, Europe and the British Isles (Hutkins, 2006; Ornelas & Kiple, 2000).

Throughout history, there is no doubt that fermented foods have been among the most important foods consumed by humans. These products contributed directly to the cultural and social evolution mankind's history by simply being integrated in a lot of civilizations diets and cuisines or consumed as part of religious customs, rites and rituals (Hutkins, 2006).

2.1.1.1. The discovery of the Science behind fermentation

It was hard for people in the past to know what happened when food fermented. The early manufacturers of fermented food and beverages didn't know the actual science behind it because the microorganisms and enzymes would only be "discovered" in the last 200 years.

In 1675, Antonie van Leeuwenhoek, with the help of his crude microscope, discovered what he would call later "animalcules". But the relationship between these "animalcules" and fermentation would only be discovered one century later (Barnett, 2003; Hutkins, 2006).

In the late 1700s and early 1800s, chemists began to study fermentation. Lavoisier and Gay-Lussac discovered the equations for the alcoholic fermentation. With the improvements in microscopy, Kützing and Schwann observed the presence of yeast cells in fermenting liquids, including beer and wine. In 1837, Schwann proposed that with the development of fungus would mean that the fermentation had started. It was only in 1857, that a chemist named Louis Pasteur concluded that fermentation was not a lifeless phenomenon but rather a living process, or in other words, fermentation would only happen when microorganisms were present (Barnett, 2003; Hutkins, 2006).

Once the scientific basis of fermentation was established, people started channeling their efforts to identify and cultivate microorganisms capable of helping with fermentations. Breweries, such as the Carlsberg Brewery in Copenhagen, started using pure yeast strains, based on the recommendations made by Pasteur and others (Barnett, 2003; Hutkins, 2006).

2.1.2. Types of fermented foods

2.1.2.1. Fermented vegetables

Vegetables are part of every kind of diet and are often eaten as salads, curries, pickles or soups. Some vegetables, however, suffer from a major setback – they are easily perishable. Throughout the world, many techniques were developed to increase the shelf-life of food products such as refrigeration, freezing or canning. Sadly, in poor and developing countries, these techniques are not affordable, so they rely on natural fermentation as a mean of preservation. Acid fermentation with salting remains as one of the most practical methods to preserve fresh vegetables and increase its organoleptic and nutritional quality. Fermented vegetables are acidic in nature, which means they are produced mainly by lactic fermentation.

One the biggest examples of a fermented vegetable is *sauerkraut* (sour cabbage). To prepare this dish, white cabbage is shredded finely and layered with salt in large crocks or wooden tubs. These are then covered with a heavy lid and left to ferment, below 15.5ºC for about 1 month. It's usually eaten together with smoked meats or sausages or even on its own (Kailasapathy & Tamang, 2010).

Another big example of a fermented vegetable is the *kimchi*. *Kimchi* is a generic term used in Korea to classify a group of fermented cabbage, radish and garlic foods. Without starter cultures, *kimchi* is made through lactic fermentations of Chinese cabbage at low temperatures to ensure proper ripening and preservation. It is made by cutting cabbage and radish into small chunks and salting them. Spices like garlic or leaf mustards are also added. Some fermented fish is then boiled and cooled down before adding to the mixture. Store it inside small pots and wait until the fermentation is complete **(Kailasapathy & Tamang, 2010)**.

2.1.2.2. Fermented soybeans and non-soybean legumes

Legumes are high-protein foods consumed in the human dietary system. Some common examples are soybean, black lentil, garden pea, etc. Fermentation of soybean-based products account for almost 90% of the fermented legumes consumed. Fermentation of soybeans is an ancient method used mostly in Asian countries, such as China, Japan, Korea or Thailand. In Africa, however, fermentation of non-soybean legumes is more common. Sometimes a starter culture of *Bacillus Subtilis* is added to these fermented legumes in many Asian and African countries. Fermented legumes are alkaline in nature because of alkaline fermentation.

Fermented soybeans foods are often divided in two small groups: those fermented by *Bacillus spp.* and those fermented by moulds. One of the examples of fermented soybean foods fermented by *Bacillus spp.* is *natto*. *Natto* originates in Japan and possesses a characteristic ammonia odor. It is prepared by soaking the soybeans overnight and then boiling them. These soybeans are then wrapped in rice straws that were sterilized previously with boiling water. The fermentation takes around 2 days and the result is a sticky viscous paste which is eaten without frying or any cooking at all (Kailasapathy & Tamang, 2010).

The biggest example of a fermented soybean food fermented with mould is *tempeh*. Even though the origin is still unknown, it is said that this food came either from coconut tempeh produced in Indonesia or from the method of production of fermented soybeans with *Aspergillus*, but this mould was replaced with *Rhizopus oligosporus* in Indonesia because of the environmental suitability. To make *tempeh*, in Indonesia, soybeans are soaked in water and after being dehulled and drained they are inoculated with a tempeh starter (*Rhizopus* spores developed on hibiscus leaves). The inoculated soybeans are then packed either in banana leaves or plastic bags and fermented at 30ºC for 24 hours (Kailasapathy & Tamang, 2010; Okada, 1988).

One example of a fermented non-soybean legume food is *Dawadawa*. This ethnic fermented food is commonly prepared in West Africa from locust beans. To prepare this food, locust beans are boiled for 24 hours and the seed coats are removed. The beans are boiled a second time and then a softening agent is added for 1 or 2 hours. The beans are spread on a calabash tray and covered with a cloth. The fermentation happens naturally for 2 up to 4 days (Kailasapathy & Tamang, 2010).

2.1.2.3. Fermented milks

Milk is a globally known drink. It's a polyphasic emulsion that have physical, chemical and biological properties. Fermented milk products are prepared from whole milk, partially/full skimmed milk, or concentrated milk by microbial fermentation through the use of lactic acid bacteria. Fermented milk is widely accepted by consumers because of its taste and its extended shelf-life. Lactic acid bacteria convert the lactose of milk into lactate and the proteins into free amino acids while also promoting the rapid lowering of the pH (acidification). This acidification allows the product to increase its shelf-life by inhibiting spoiling with pathogens and microorganisms. Some commonly known examples of fermented milks products are cheese and yogurt.

One example of a fermented milk product less known but that is starting to rise in popularity is the *kefir*. There is not a definition of what *kefir* is but it's a viscous, acidic and mildly alcoholic milk beverage produced by the fermentation of milk with a kefir grain as the starter culture (FAO/WHO, 2003). These *kefir* grains have the appearance of a cauliflower and a firm texture. The *kefir* grain possesses an inert polysaccharide matrix with a relatively stable and specific microbial community composed of lactic acid bacteria, acetic acid bacteria and yeasts which coexist in a complex symbiosis. After the fermentation is complete, the grains can be used for another fermentation. At home, *kefir* is produced by incubating milk with *kefir* grains at 20-25ºC for 18 to 24 hours. At the end of fermentation, the grains are rinsed several times with water and then transferred to initiate a new batch. Industrially, *kefir* is produced through a starter with *kefir* grains which is used to inoculate a second batch of *kefir* that constitutes the actual commercial product (Kailasapathy & Tamang, 2010; Simova, et al., 2002).

2.1.2.4. Fermented fish

Fish has always been a common ingredient present in the diets of people who live near lakes, rivers and coasts. Fish, though, is a very perishable product, so several conservation methods like salting, drying or fermentation have been used by people. Fermented fish products contribute significantly to the diet by increasing the protein intake of a lot of people in the world (Beddows, 1985). Fermenting fish is usually prepared at home and is used to prepare mostly fish sauce and can be consumed as staple food, side dish or condiment in Asia.

Fermented fish products are prepared from freshwater and marine finfish, shellfish and crustaceans that are processed with salt to cause fermentation and prevent putrefaction (Ishige, 1993; Kailasapathy & Tamang, 2010).

2.1.2.5. Fermented meat products

Animal meat is consumed throughout the whole world except by a majority of Hindus and some other communities because of their religious beliefs. Meat is a highly perishable product and can be an agent for the transmission of a range of infections and intoxications. Due to this

reason mankind, early, started developing techniques like drying, salting or fermenting to preserve this kind of product.

Fermented meat products are divided into two categories: those made from whole meat pieces or slices, such as dried meat or jerky, and those made by chopping the meat, usually called sausages. Meat processing is the combination of chemical curing, fermentation and drying, which combined give stable, safe and ready to eat meat products (Bacus, 1984). Fermented sausages are made from chopped or ground meat that is mixed with other non-meat ingredients such as curing salts or spices and undergo a lactic fermentation in the course of the drying process.

To prepare a fermented meat product, in theory, any type of animal meat would suffice. There are reports of exotic types of meat like deer or ostrich (Capita, Llorente-Marigómez, Prieto, & Alonso-Calleja, 2006) but pork, beef and poultry are the most common. The meat and the fat are minced or chopped prior to mixing with the other ingredients in a bowl chopper. These operations are best performed at low temperatures (about 4ºC) to avoid the mixing of the meat and fat particles. The curing salts used are most commonly a mixture of two or three different salts and are very important in the fermentation process because they ensure that the product has the desirable texture, flavor and color (Kailasapathy & Tamang, 2010).

Once the ingredients are mixed, they are packed into the casing which is traditionally made from the collagen of the intestinal tract of the animals used. Recently, these casings have been replaced by a regenerated collagen casing produced from the corium layer of cattle hides due to their better permeability and adherence to the sausage mix (Kailasapathy & Tamang, 2010).

2.1.2.6. Other fermented products

There are some fermented products that do not into the previous categories. These products are for example vinegar, tea, coffee, cacao, etc. Vinegar has been used as a condiment, a preservative and a medicine since ancient times. Vinegar can be prepared from any substrate containing sugar and hydrolyzed starchy materials through alcoholic fermentation followed by acetic fermentation (Yokotsuka, 1991).

Tea is originated from China and is the second most popular beverage in the world. It's generally produced by a natural oxidation process without the involvement of any type of microorganism. However, there are a bunch of microbial fermented teas like *kombucha* from China or *miang* from Thailand. *Kombucha* is a slightly carbonated tea beverage consumed throughout the whole world but that, like said before, was originated in China. It is prepared by fermenting black tea that has been sweetened with sugar. To make this beverage, black tea leaves are infused in boiling water sweetened with sucrose for about 10 minutes. After the leaves are removed, the tea is poured into a jar and acidified by the addition of vinegar or tea fungus (a floating cellulose mat formed by a symbiotic association of yeasts and acetic acid bacteria from a previous fermentation. This jar is then covered with a clean cloth and this preparation is incubated for ten to twelve days. The final product comprised organic acids, vitamins, minerals and tea components, resembling the taste of cider (Greenwalt, Steinkraus, & Ledford, 2000). *Kombucha* has gained popularity

because of the health benefits resulting from regular consumption. Health benefits like stimulation of the immune system, digestion, liver function improvement, reduction of obesity or detoxification activity are some examples but little to no studies have proved this is right (Kailasapathy & Tamang, 2010).

The name of coffee is derived from the word *Kaffa* from the southwestern province of Ethiopia. There are wild coffee plants spread all over the African continent which indicates that this plant originated from here. From Ethiopia, coffee spread to Arabia where the coffee beans started getting roasted and brewed. Coffee seeds are harvested from the coffee trees and are either processed by either wet or dry methods to remove the pulp and the material that surrounds the seeds (Silva, Batista, Abreu, Dias, & Schwan, 2008).

In the wet process, the coffee cherries are hand-picked and de-pulped with the help of machines, which removes all the extraneous material and leaves the beans surrounded by the parchment and a layer of mucilage. These are then sorted by density and put in cement tanks together with water and are allowed ferment. The main goal of this fermentation is to degrade the mucilage adhering firmly to the coffee beans. This fermentation is may last from 24 to 48 hours for best quality production. After fermentation, the beans are removed from the tanks, washed and left to dry for about 1-2 weeks (Kailasapathy & Tamang, 2010).

In the dry method, however, things are simpler. This kind of method is mainly used with the Robusta coffee which has a thin pulp that allows direct drying (Fowler, Leheup, & Cordier, 1998). In Brazil and Ethiopia, though, Arabica coffee is also processed through sun drying. The coffee cherries are spread on drying grounds or mats in layers and are exposed to sun. The time of drying depends on the sun shine and may vary from 3 to 4 weeks (Kailasapathy & Tamang, 2010).

As a summary, table 2.1 represents the aforementioned products regarding the raw materials used, the use (or not) of starter cultures and some health benefits.

Table 2.1 – Summary table of the fermented products previously mentioned as well as their raw materials, starter cultures and some health benefits.

Products	Raw materials	Starters	Some health benefits	References
Sauerkraut	White cabbage	-	Reduced risk of tumors	Tamang, 2017 Otes, 2003
Kimchi	Cabbage, radish or garlic	-	Antiobesity effect, antioxidant and antistress activity and reduced risk of tumors	Tamang, 2017
Natto	Soybeans	*Bacillus spp.*	Gum swelling prevention, hypertension reduction, protection from osteoporosis	Tsubura, 2012 Tamang, 2017

Tempeh	Soybeans	*Rhizopus oligosporus*	Prevents hyperlipidemia, cancer (breast and colon) and cognitive decline	Kiriakidis, 1997
Dawadawa	Locust beans	-	High content in proteins	Esenwah, 2008
Kefir	Milk	*Kefir* grains	Tuberculosis and cancer treatment, antiallergic properties	Tamang, 2017
Fermented fish	Fish and shellfish	-	Lowers the risk of cardiovascular diseases and antiallergic effect	Tamang, 2017
Sausages	Pork, beef, poultry, deer or ostrich	-	Prevention of gastrointestinal disorders	Tamang, 2017
Kombucha	Tea	Yeasts and acetic acid bacteria	Probiotic and antiallergic effects	Tamang, 2017
Coffee	Coffee seeds	-	Prevention of cardiovascular diseases, Parkinson's disease, Alzheimer's disease and cancer	Butt, 2011

2.1.3. Health benefits of fermented foods

Fermented foods have an immense range of health benefits. They possess functional and therapeutic values by having antioxidant, antimicrobial, low-cholesterol, essential amino acids, and some important bioactive and health-benefit compounds which are considered as sources of medical therapy for humans. Fermented foods also have a role in the prevention of many diseases and health problems (figure 2.1).

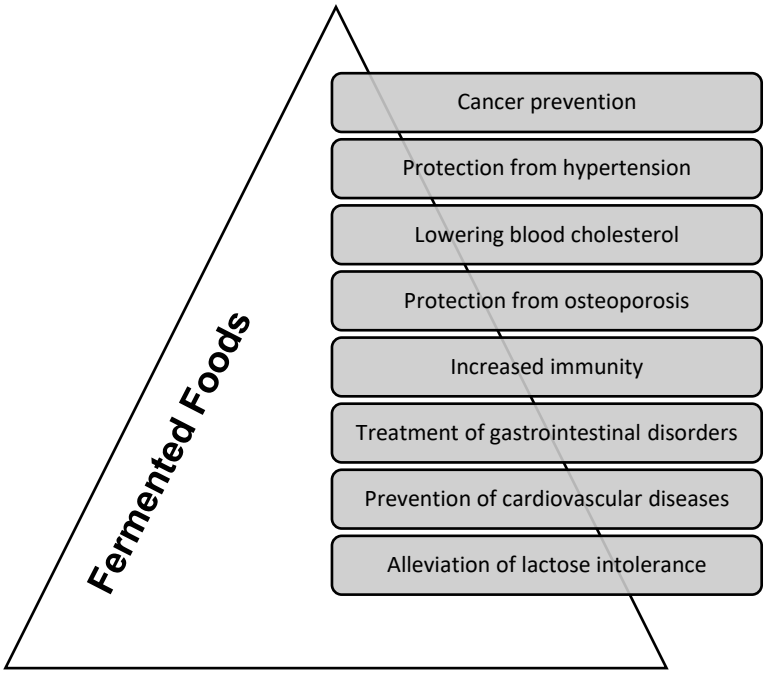

Figure 2.1 - Some health benefits of fermented foods. (Source: Adapted from Fermented foods and beverages of the world, 2010).

Some fermented foods are being reported for having cancer prevention properties. For example, fermented red beet prevents the proliferation of tumor cells. The fermentation of vegetables produces lactic and acetic acids and make them nutritious, palatable and wholesome fermented foods. Lactic acid is important because it protects the body against several infections and liver diseases, as well as improving the digestion and increasing immunity by protecting the body from some infectious agents (Karovicova & Kohajdova, 2005).

Kefir is a fermented beverage with a lot of health benefits. It is easily digestible, and it provides beneficial bacteria and yeast, vitamins, minerals and proteins contributing to a healthy immune system and helping patients suffering from AIDS, chronic fatigue syndrome and cancer. It used to be used as a treatment for tuberculosis and cancer when modern medical treatment wasn't available (Otes & Cagindi, 2003).

Fermented cabbages, cabbage juice and sauerkraut are also related to cancer prevention because they have in their constitution a molecule called s-methylmethionine, which reduces the

risk of tumorigenesis in the stomach. Cabbage by itself contains isothiocyanins that are responsible for anticancer effects in cancer of the colon, breast, lung and liver (Kris-Etherton, et al., 2002).

Natto is a popular fermented product in japan for more than 400 years made from soybeans cultured with *Bacillus subtilis*. This fermented food contains saponin and isoflavones, vitamin K_2, and dipicolinic acid, which are generated from both soybeans and *natto* itself (Hosoi, 2003). After the start of fermentation by *natto* bacteria, the concentration of vitamin K_2 increases to 124 times that in the soybeans itself (Yanagisawa & Sumi, 2005). This vitamin stimulates the growth of bone, which might help prevent osteoporosis in older people in Japan.

Moderate consumption of wine is often associated with the prevention of cardiovascular diseases. Some studies (Klastky, Armstrong, & Friedman, 1997; Renaud & Lorgeril, 1992) report that drinking alcoholic beverages, specially wine, at a moderate level of two drinks per day may have protective effects and cardiovascular benefits.

Bifidobacteria are a natural inhabitant of the human large intestinal tract, and it has reported that large numbers of *Bifidobacterium* form a barrier against pathogens by controlling the intestinal pH level through the release of acetic and lactic acids (Lauer & Kandler, 1976). There's also another study that report that these bacteria stimulate an immune response of the host (Sekine, 1985). Thanks to these studies, *Bifidobacteria* have been incorporated in several types of fermented foods like yogurts, fermented milk or animal feed additives.

These examples show some health benefits of some fermented foods and these benefits are expressed either directly through the interaction of ingested live microorganisms, such as bacteria or yeasts, or indirectly through the ingestion of metabolites produced during the fermentation process. Fermented food therapy also has been applied to a wide range of health disorders, such as gastrointestinal disorders. Fermented foods that possess such an impact on health, will remain an important food in the years to come (Kailasapathy & Tamang, 2010).

2.2. Lathyrus sativus L.

The grass pea (*Lathyrus sativus* L.) has gained interest as a plant. It adapted to arid conditions and contains high level of protein, a component that has been hard to obtain is certain developing areas. Its plant is a low-growing vine with small blue flowers (figure 2.2A). The genus *Lathyrus* is large with 187 species and subspecies being recognized with species being spread among the Old and New World. However, only 1 species – *Lathyrus sativus* – is widely cultivated as a food crop, while other species are cultivated less for both food and forage. Grass pea belongs to the family Leguminosae (=Fabaceae), subfamily Papilionoideae, tribe Vicieae (Campbell, 1997).

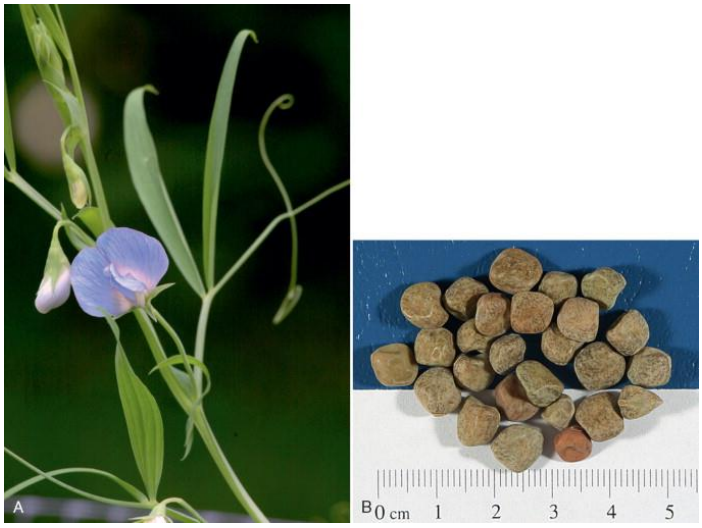

Figure 2.2 – *Lathyrus sativus* flowers (A) and *Lathyrus sativus* seeds (B) (source: Dobbs, 2009).

Grass pea is an important crop of economic significance in India, Bangladesh, Pakistan, Nepal and Ethiopia. It is cultivated and naturalized in Central, South and Eastern Europe (from Germany to Portugal and Spain, east to the Balkans and S. Russia), Cyprus, West Asia and North Africa (Campbell, 1997).

The grass pea is endowed with many properties that combine to make an attractive food crop in drought-stricken, rain-fed areas where soil quality is poor and extreme conditions prevail. Despite its tolerance to drought, it is not affected by excessive rainfall and can be grown on land subject to flooding. Its root system can penetrate any type of soil, so it is able to grow in very poor soil and heavy clays. Thanks to its intrinsic ability to fix atmospheric nitrogen, grass pea has been an attractive crop for adverse agricultural conditions (Campbell, 1997; Grela, Rybinski, Klebaniuk, & Matras, 2010).

2.3. Miso

2.3.1. Brief history

Miso was originated in China and it was introduced in Japan 1300 years ago by Buddhist monks (Tasty, 2018). It is believed that today's miso is the descendant of a Japanese ancient food called *hishio*. *Hishio* was made from fish and salt at the beginning of Japanese history. The fish tissues were degraded by the enzymes existing inside the organs when a high salt concentration was present. Fish as a raw material of *hishio* ended up being gradually replaced by beans and grains such as soybeans or wheat, thanks to the influence of Buddhism that has a principle of abstaining from eating any kind of fish or meat (Hui, Meunier-Goddik, Josephsen, Nip, & Stanfield, 2004; Wood, 1985).

In Japan, during the 8th-12th century, miso was considered a delicacy and would only be eaten by the nobility and monks mainly because it was made using rice. Peasants were forbidden to use the rice they harvested to make their own miso, so they started using alternative grains such as barley. During the Muromachi era (13th-16th century), the monks created a new method of making miso which consisted in grounding the beans instead of using them as a whole, so it started being consumed as a paste (Miso, n.d.).

2.3.2. Types of miso

The most common classification divides miso into two basic categories:

i. **Regular miso** – consisting of rice, barley and soybean miso. It is used mainly in cooking and, in japan, particularly in soups;

ii. **Special miso** – consisting of *namémiso* and sweet simmered miso. It isn't cooked, and it is used mainly as a topping for grain dishes, fresh vegetable slices and tofu;

Each type of miso inside these three categories have their own kind of unique varieties. They all differ in the proportions of the ingredients used, the cooking methods used and the duration/temperature of fermentation. In recent years, the number of miso varieties have been increasing significantly.

Before digging into the different varieties of miso there are some concepts that are important to be explained. It is also important to note that all varieties of miso share six principles attributes: method of fermentation, flavor, color, texture, cost and place of origin.

Natural miso vs. Quick miso: Natural miso is prepared in a traditional way and has three basic characteristics: slow fermentation (usually for six months up to three years), made only using natural ingredients without any chemical additives and it isn't pasteurized.

Quick miso is a product that started being produced in the modern days. It is about 25% cheaper and is fermented for a short amount of time (usually for about three months) in a temperature-controlled environment. Since the fermentation is so short, the product doesn't have the

ability to develop the flavor, the aroma and the color that are inherent to natural miso. There is a need to add chemicals and synthetics to mimic natural miso characteristics. This miso needs to be pasteurized to prevent microorganisms from producing carbon dioxide which would cause the plastic bags to explode. This pasteurization also hurts miso's flavor and aroma (Shurtleff & Aoyagi, 1983).

Salty miso vs. Sweet miso: All misos can be grouped according to their salt content: 11% to 14% of salt content is considered salty miso, 7% to 11% of salt content is considered mellow miso and up to 7% of salt content is considered sweet miso.

In Japan, the sweetest misos are found in Kyoto. As one moves from modern urban areas to more traditional areas, there is an increase of saltiness in misos. Sweet misos are usually picked by office workers, young people and the upper classes while the saltier misos are picked up by farmers and elderly adults. There has been an increase of sweet miso consumption since the end of World War II, colliding with Japan's rapid urbanization and industrialization.

Sweet miso is mostly used in toppings, sauces and vegetable side dishes while salty miso is usually used in soups and with seafoods. Because the natural sugars tend to ferment quite easily, forming alcohols, sweet miso has a short shelf life and therefore really hard to be exported. On the other hand, salty miso can be stored for as long as people want even at room temperature (Shurtleff & Aoyagi, 1983).

Red miso vs. White miso: All misos can be divided in red misos (in the range of brown) and white misos (soft yellow-beige range). Most red misos get their color naturally through lengthy aging, whereas white miso is prepared by a temperature-controlled and quick fermentation. In general, rice miso tends to be lighter colored than barley miso, and barley miso tends to be lighter than soybean miso.

The great majority of white miso is made using rice *koji* and contains a large amount of carbohydrates and little to no salt and that's why most white miso is rather sweet (Shurtleff & Aoyagi, 1983).

With some concepts already explained, it is now possible to talk about the basic categories mentioned previously:

Regular miso

The three basic types of miso -rice, barley and soybean- are classified according to the raw material used or the substrate for *koji*. There is a preference in the production of rice miso in Japan (about 81%) while barley miso (11%) and soybean miso (8%) are less preferred. Each of these three types can then be divided according to their flavor (sweet, mellow and salty) and their color (red, lighty-yellow and white) (Shurtleff & Aoyagi, 1983).

Rice miso (*komé miso*)

Rice miso accounts to up to 81% of the miso sold in Japan. Very rich in glucose and other natural sugars, rice serves as the basis to make *koji* used in most of sweet, quick and white misos. Saltier misos have been commonly produced in cold provinces north of Tokyo and are noted for having the highest quality of rice (Abiose, Allan, & Wood, 1982; Shurtleff & Aoyagi, 1983).

Red miso (*Aka* miso): The fermentation time of this miso is from one year to up to 3 years, or for three to four months in a temperature-controlled environment. Red miso possesses a rich and savory salty flavor with subtle sweet undertones. Its color ranges from russet to dark reddish brown and its texture can range from chunky and soft to smooth and firm (Lewin, 2018).

Red miso has the lowest proportion of carbohydrates (19,1%), the second highest proportion of proteins (13.5%) and the highest proportion of salt (13%). Therefore, red miso can be stored for several years at room temperature.

Sweet red miso (*Edo miso* or *Edo Ama-miso*): It has a slightly savory aroma and a deep, mellow sweetness. It contains a large amount of carbohydrates (32%) and a low amount of both salt (6%) and proteins (12,7%). It ranges from light red-brown to russet and it has a chunky texture. This kind of miso is fermented during ten to twenty days during the summer and four to five weeks during the winter. A fairly good approximation of this type of miso's flavor can be achieved by mixing three parts of red miso with one part honey.

Light-Yellow miso (*Shinshu* Miso): *Shinshu* was the name of the ancient province in Japan – now Nagano prefecture – where this kind of miso was first developed. Modern *Shinshu*, which we now call light-yellow miso, is a quick miso with a mature and mellow salty flavor. This kind of miso has a light and refreshing aroma and its color scheme ranges from light yellow to brown-yellow. Low in carbohydrates (19,6%) and quite high in salt (12,5%) and protein (13,5%), it is kept for two months at room temperature, and has to be refrigerated incase of longer storage.

Sweet miso (*Shiro miso* or *Saikyo Miso*): Sweet white miso is made by combining four parts by weight of rice *koji*, two parts of soybeans and one part of salt. It's very sweet and smooth and can be spread in pancakes or bread like butter. It ranges in colors from ivory to light yellow.

It contains the highest amount of carbohydrates of all misos (36%) and the lowest in both salt (5,5%) and proteins (11,1%). Its abundance in natural sugars boosts the fermentation process to three weeks, becoming as short as one week in the summer and about one to two months during the winter. Since this miso can be prepared very quickly, it is an excellent variety to be prepared at home. Its flavor can be obtained by mixing two parts by volume of light-yellow miso, one part honey and one part water.

Barley miso (*Mugi miso*)

Barley miso is generally darker, saltier and ages longer than rice miso. Generally sold in chunky or *koji* form, its unique texture is the characteristic that the customers prefer the most. The

koji is prepared from either polished of pearled barley which is higher in protein and lower in carbohydrates than polished rice. Therefore, barley miso is usually not as sweet as rice miso and takes longer time to ferment (Abiose, Allan, & Wood, 1982; Lewin, 2018; Shurtleff & Aoyagi, 1983).

Barley miso (*Karakuchi Mugi miso*): Despite having the same name as its large category, this product is usually the one referred in all recipes. Its relative high salt content (13% or more) is mellowed by the barley's subtle sweetness. It has a low amount of carbohydrates (21%) and high amount of proteins (13%) and it's fermented naturally by about one year. By the end of that time, it acquires a red-brown color, chunky texture and a prominent fragrance. When aged for three years, its color turns into chocolate brown, its texture becomes more homogenous and the flavor grows richer and subtler.

Soybean miso (*Mamé miso*)

Soybean miso is different than rice and barley miso because it contains no grain and its *koji* is made exclusively from soybeans. Due to the lack of carbohydrates and the high content in salt, soybean miso takes more time to ferment. Since it's impossible to vary the ratio of grains in the basic ingredients, the several varieties have a low range of flavors and aromas. All of them are fermented for a year (Abiose, Allan, & Wood, 1982; Lewin, 2018; Shurtleff & Aoyagi, 1983).

***Hatcho* miso:** It has a savory aroma, deep mellow sweetness and a unique astringent flavor, reminiscing of chocolate. Dark cocoa brown and with a chunky texture, *hatcho* is so firm that's it's possible to cut it with a knife. Higher in protein (21%) and lower in carbohydrates (12%) and water (40%) than any other miso, it still contains a high amount of salt (13%).

To make *hatcho* miso, a unique species of mould is used, replacing *Aspergillus oryzae* for *Aspergillus hatcho* which flourishes in the Okazaki area. The fermented soybean *koji* is mixed with salt and a small amount of water and then packed into 200 years old cedar vats to ferment for up to 3 years.

Soybean miso (Mamé miso or *Ichi-nen Mamé miso*): It is also called "one-year" or "regular soybean miso so it can be distinguishable from other soybean misos. It is prepared like *hatcho* miso, but the minimum aging requirement is one year instead of two years and the usual mould used to make *koji* is *Aspergillus oryzae*. Comparing to *hatcho*, this kind of miso possesses a redder color, it's less rich and has a softer texture thanks to its water content (48%).

Special miso

Special misos are very different from the rest of the misos mainly because:

1. In addition to the normal ingredients (soybeans, *koji* and salt), it's also added chopped vegetables, nuts, seeds, seafoods or natural seasonings;
2. Usually sweet and, if not refrigerated, have a short shelf life;
3. Normally used as toppings or seasoning and never in miso soups;
4. Sold in small quantities (200 grams).

The two basic types are *namémiso* and sweet simmered miso.

Namémiso: This type of miso is prepared by fermenting a small amount of chopped vegetables and some spice seasonings (≈10%) with whole cooked soybeans (15%) and a large amount of whole-grain *koji* (75%) usually containing barley or wheat. The general consistency achieved is the same as apple sauce but a little less chunky, and the color is usually light brown. The high proportion of carbohydrates, which are broken down into sugars, give this miso its characteristic sweetness and delectable aroma (Shurtleff & Aoyagi, 1983).

Sweet simmered miso: This type of miso is prepared by combining regular miso with a mixture of sweetening (sugar or honey), a little water or sake, nuts, seeds, minced vegetables or seafoods. The mixture is cooked and stirred constantly until it acquires the same degree of firmness of regular miso. The most widely available commercial variety is peanut miso, which contains peanuts, roasted sesame seeds and in some cases raisins.

2.3.3. Miso's microbiota

Microorganisms play an important role in food fermentation mainly because they determine the characteristics of the food in terms of acidity, flavor and texture but as well as the health benefits that go beyond nutrition. These microorganisms may be inherent of the product's microbiota or as a result of an intentional addition of starter cultures. This addition is mainly to produce several compounds (enzymes, flavors, etc.) specifically to act as food additives or as part of the fermentation processes.

The main types of microorganisms present on miso's microbiota or any kind of fermented food are bacteria, moulds and yeasts.

Bacteria are the most dominant microorganism appearing in both naturally fermented foods or fermented foods with the addition of a starter culture. Among the bacteria, lactic acid bacteria are the most common type of bacteria to be present in fermented foods. Non-lactic acid bacteria are also present in food fermentation but normally as minor or secondary groups. Lactic acid bacteria transform simple sugars into various organic acids, like lactic acid, and other anti-microbial substances that inhibit the growth of harmful bacteria thereby prolonging its shelf life. The

main bacteria groups present in miso are *Lactobacillus* (*delibrueckii*) and *Pediococcus* (*halophilus*) (Shurtleff & Aoyagi, 1983; Tamang, 2017).

Yeasts form an inevitable part of the microflora of fermented foods. Their principal role in food fermentation is to ferment sugar, to produce secondary metabolites, to inhibit the growth of moulds that produce mycotoxins and to show several enzymatic activities such as lipolytic, proteolytic or glycosidasic. Yeasts react with sugar to produce alcohols contributing to the miso's aroma. Besides producing alcohol, they also produce proteins, vitamins and minerals playing a very important role on the enrichment of fermented foods. The main yeast groups present in miso are *Candida* and *Zygosaccharomyces* (Montet & Ray, 2016; Tamang, 2017).

Moulds' main role, most commonly as filamentous moulds, in fermented foods are the production of proteolytic and lipolytic enzymes. Moulds initiate the degradation of the raw material through this ability to produce these enzymes. The resulting product (*koji*) serves as the starting point for fermentation by bacteria and/or yeasts for further processing. Moulds also play an important role on the flavor and texture of the final product. The main mould group present in miso is *Aspergillus* (Gow, 1995; Tamang, 2017).

2.3.4. How is miso made?

The word miso means "fermented beans" in Japanese. Miso is a savory, high protein seasoning made from soybeans, rice, salt, water and *Aspergillus oryzae* culture. There are different methods of making miso depending on the variety of the soybean, but the basic process is essentially the same every time. For example, to make Japanese rice miso there are five simple steps: rice *koji* preparation, treating soybeans, mashing and mixing of all ingredients, fermentation and pasteurization and packaging (Hui, Meunier-Goddik, Josephsen, Nip, & Stanfield, 2004; Liu K. , 2004; Shurtleff & Aoyagi, 1983).

Rice *koji* preparation. Non-glutinous rice is cleaned, washed and soaked overnight and then it's steamed for about 40 minutes. The rice is then cooled to a temperature of about 35ºC and inoculated with a *koji* starter containing *Aspergillus oryzae* spores. The next step is to incubate at a temperature between 30ºC and 35ºC with a relative humidity percentage higher than 90%. After about 40 hours of inoculation, when the cooked rice is completely covered by a white mycelium, it becomes a fermented mass known as *koji*.

a. Treating soybeans. The soybeans are cleaned, washed and soaked on water overnight. In the next day, they are cooked in boiling water.

b. Mixing and mashing all the ingredients. After the soybeans are cooled to room temperature, they are mixed with rice *koji* and water containing the inoculum, which may be from a previous batch or a pure culture.

c. Fermentation. After everything is mashed and mixed, this mixture is packed into pots or jars. The miso will then ferment at a controlled temperature, normally between 30ºC and 38ºC for a period of about 6 months, depending on the type of miso.

d. Pasteurization and packaging. When the miso is ripe, it's blended and mashed again. After it gets pasteurized, some preservatives might be added, the miso is then stored either in bags or cubic containers, so it can be sold. The final product, a paste-like product, is used to make miso soup simply by diluting it with water and adding several kinds of vegetables, chicken or other type of meat and fish.

2.3.4.1. *Koji* production

The word *koji*, in Japanese, describes a fermented mass made from growing moulds either on rice, barley, wheat, soybeans or a combination of them. In its composition, *koji* contains a large variety of enzymes that digest starch, proteins and lipidic components found in the raw materials. *Koji* can be used to make soy sauce, fermented soy paste (miso) and Japanese sake.

The first fermentation process occurs when the "*koji* starter" (consisting of dried mould spores or dried *koji*) is mixed with cooked rice, barley or soybeans. This mixture is then incubated and allowed to ferment for about two days to produce mould grains or soybeans, called *koji*, rice covered by a white mycelium (Hui, Meunier-Goddik, Josephsen, Nip, & Stanfield, 2004; Shurtleff & Aoyagi, 1983).

The *koji* starter, also known as seed *koji* or *tane-koji*, provides spores of microorganisms so it's possible to make it. These microorganisms that are found in *koji* starter are always moulds and they can be either *Aspergillus oryzae* or *Aspergillus sojae*. This mould is the one that produces the enzymes that later will break down the complex molecules of proteins, starch and lipids, making them more digestible for the bacteria and yeasts to ferment them. For example, in the process of making beer, malt is prepared by sprouting barley (without the use of any mould) so it can develop enzymes. Thus, *koji* is to miso what malt is to beer (Hui, Meunier-Goddik, Josephsen, Nip, & Stanfield, 2004; Shurtleff & Aoyagi, 1983).

Aspergillus sp. only reproduce asexually, and have the ability to use starch, oligosaccharides, simple sugars, organic acids and alcohols as a source of carbon. They can also use proteins, amino acids and urea as a source of nitrogen. These types of mould are aerobic, meaning they only grow in environments that possess oxygen, with an optimal growth at pH of 6.0, a temperature of 37ºC. When the air supply is limited, its growth rate decreases. When the temperature is below 28ºC, the enzymatic activities remain high but the growth rate decreases (Hui, Meunier-Goddik, Josephsen, Nip, & Stanfield, 2004; Shurtleff & Aoyagi, 1983).

In the traditional way to make *koji*, the inoculated mixture is put in wooden trays and stored for three or four days in a room. When the mould is growing, the temperature and moisture are controlled by manual stirring. However, in modern times, the inoculated mixture is put inside a

perforated vat where the air is circulated, and temperature and humidity are controlled. After three of four days, the mixture turns green-yellow thanks to the mould sporulation and so it becomes matured *koji* (Hui, Meunier-Goddik, Josephsen, Nip, & Stanfield, 2004; Shurtleff & Aoyagi, 1983).

In the process of making *koji*, there needs to be a preoccupation of cooling the materials twice either by hand or with a help of a mechanical device, when their temperature increases to above 35ºC because of the mould growth. In the beginning phase of *koji* making, a temperature between 30ºC and 35ºC is advisable for mycelium growth but also to prevent the growth of *Bacillus* as a contaminant. In the latter stages, a lower temperature (20-25ºC) is needed in order to allow the maximum production of enzymes (Hui, Meunier-Goddik, Josephsen, Nip, & Stanfield, 2004; Shurtleff & Aoyagi, 1983).

The inoculated substrate is placed in a large cloth-lined box called a crib, covered with thick insulating mats, and allowed to stand overnight. After some hours, the temperature inside the crib starts increasing thanks to the heat generated by the fermentation process. In the next day, the ingredients have to be stirred in order to replenish the oxygen supply, release some carbonic gases, and to mix the warmer substrate in the center with the cooler substrates at the top, bottom and the sides.

To protect against the contamination of microorganisms, all the tools and hands must be perfectly cleaned and *koji* needs to be kept within the required range of temperature and moistness. If the temperature of *koji* rises above 40ºC, the production of enzymes decreases, and undesirable microorganisms begin to appear. If the temperature rises above 45ºC, *koji* starts to die thanks to its own production of heat (Hui, Meunier-Goddik, Josephsen, Nip, & Stanfield, 2004; Shurtleff & Aoyagi, 1983).

If the temperature and the humidity are kept within the ideal range, only *Aspergillus oryzae* moulds propagate. In order to prevent *koji* of overheating, to remove carbon dioxide and provide a new oxygen source, and to help the mould penetrating better into the substrate, the mycelium is broken up and stirred at regular intervals. After 40 to 50 hours, *koji* must be harvested while the conidiophores of the mould are still forming, before the typical green color of sporulation appears.

The energy required to grow and support moulds and create the needed enzymes is supplied by the substrate, either grain or soybeans. During the first fermentation, about 5 to 10 percent of the substrate is consumed by the mould, so the finished miso loses some nutritional value.

As the process of making *koji* finishes, it is time to cook soybeans. Soybeans are boiled or steamed until they are soft enough to be crushed easily by your fingers. They need to be cooked because this makes the proteins inside more receptive to enzyme activity and it also helps inactivating the trypsin growth inhibitors. Cooking time influences the color and flavor of the finish miso: longer cooking time means darker miso. For lighter color miso (white and yellow), the beans are cooked for 30 to 60 minutes while darker miso's cooking time is from six to eight hours. After

the beans are cooked, they are crushed to facilitate the permeation of enzymes from the *koji* (Hui, Meunier-Goddik, Josephsen, Nip, & Stanfield, 2004; Shurtleff & Aoyagi, 1983).

2.3.4.2. The second fermentation process: preparing the miso

In the second fermentation process, *koji* is mixed with cooked soybeans, salt, water and usually some mature miso. These are then packed in vats or any type of container, so the ingredients can age while the two processes of enzymatic digestion and fermentation allow the product to be transformed into finished miso.

This process is split into four stages: the mixing of the ingredients, the digestion of the protein, starch and lipids in these ingredients by the *koji* enzymes, the fermentation of these digested products by the bacteria and yeasts, and finally the aging of the miso.

The crushed soybeans are mixed with the salted *koji*, water, and "seed miso". The water is usually added so the miso can have a moisture content between 48 to 52 percent. Before adding the water, it needs to be boiled to eliminate microorganisms. A small amount of "seed miso" is added because it decreases the amount of time needed for fermentation and increases the aroma and flavor of the final miso. In modern miso production, this seed miso is replaced by the addition of a pure-culture inoculum consisting of halophilic (salt resistant) yeasts, like *Zygosaccharomyces rouxii* or *Candida versatilis,* and lactic acid bacteria, like *Pediococcus halophilus* or *Lactobacillus delbrueckii*. Before packing the mixture into the containers, they need to be cleaned and then rubbed with salt. The mixture is then added and sealed completely (Shurtleff & Aoyagi, 1983).

During the first fermentation, the amount of moulds per gram of substrate increases from 13,000 to 295,000. Since the work of these moulds is finished, the white mycelium dies from the lack of oxygen and high salinity of the surrounding environment, but the enzymes are left behind. Inside the containers, the enzymes start digesting the components of soybeans and grains. There are three enzymes active: proteases working on proteins, amylases working on carbohydrates and lipases working on lipids. The water ions react with the basic food nutrients, in a chemical reaction called hydrolysis, and produce a weak acid which allows more complex molecules to be broken down into simpler molecules that can be more easily assimilated by our bodies (Shurtleff & Aoyagi, 1983).

Proteases convert soybean protein molecules into polypeptides and peptides and then into simple amino acids. The predominant amino acid from this digestion is glutamic acid, active ingredient in a lot of seasonings. These amino acids give miso all of its flavor and some of its color, while softening and mellowing the salt present in the mixture.

Amylases reduce starches present in *koji* into simple sugars and polysaccharides, mainly glucose, but also maltose, dextrose and dextrin. These will be used by yeasts and bacteria as fermentable sugars.

Lipases transform the 18% of lipids present in soybeans into simple fatty acids, which have a variety of forms in the finished miso. Some esters of these fatty acids contribute to the final miso's aroma (Hui, Meunier-Goddik, Josephsen, Nip, & Stanfield, 2004).

As the process of enzymatic digestion comes to an end, fermentations by yeasts and bacteria starts showing predominance. Working in a sealed environment with a high amount of nutrients, the anaerobic bacteria population suddenly grow to up 100 times higher per gram of substrate. After two or 3 months of sitting in the sealed container, lactic acid bacteria assume dominance and start transforming the sugar present into several acids (especially lactic and acetic acid). These acids allow the miso to gain a little bit of sourness in the final product and also prevent spoilage (Hui, Meunier-Goddik, Josephsen, Nip, & Stanfield, 2004; Shurtleff & Aoyagi, 1983).

Soon after, some yeasts start doing their work of alcoholic fermentation. They consume sugar in order to produce alcohol (ethyl and higher varieties) and some organic acids (mainly succinic). Some yeasts produce a film on the miso's surface, while also contributing to its aroma.

As more and more substances are created by enzymatic digestion and fermentation, they start reacting with one another:

a. Organic acids start reacting with ethyl- and higher alcohols, or free fatty acids, or acetic acid to produce esters;

b. Amino acids react with sugars to produce red or brown pigments (soyamelanine), which combined with the soybean pigment and oxidation, deepens miso's color.

2.3.5. Miso's health benefits

It's hard to say what are the health benefits of miso because miso is never consumed alone as it's eaten as a topping or as soup. Soy miso is a very good source of manganese, zinc, phosphorous and copper. It's also rich in proteins, fibers and phenolic acids such as vanillic or ferulic. Miso has been used as a daily seasoning for several centuries in Japan. Since it has high content of salt, it's advised to not consume more than 6 g of miso per day. Miso has also been used to reduce hypertension, cerebrovascular disease and plasma cholesterol levels (Lewin, 2018; Tamang, 2017).

Several studies were made in order to assess some of the miso's health benefits. There is a study that wanted to know the relationship between soy products (including miso) intake and the prevalence of allergic rhinitis in pregnant Japanese women. The conclusion of this study indicated a clear inverse linear trend between the intake of dietary miso and the prevalence of allergic rhinitis (Miyake, et al., 2005). Miso is also a source of antioxidative peptides. These peptides protect the human body from free radicals and slow the progression of many chronic diseases as well as providing nutritional value (Gibbs, Zougman, Masse, & Mulligan, 2004). It has also been

proven that a diet rich in soybean products, like miso, decreases the risk of both colon and rectal cancer (Watanabe , et al., 1984).

2.4. Molecular methods for microbiota evaluation

2.4.1. Methods for identification of cultivable species

Microorganisms are key players that play an important role in ecological processes like decomposition of organic matter, soil structure formation or recycling essential elements (like carbon, nitrogen or sulfur) and nutrients. The three fundamental questions that exist while discovering and characterizing any natural or artificial ecosystem are the following

i. What type of microorganisms are present?
ii. What do these microorganisms do?
iii. How do the activities of these microorganisms relate to ecosystem functions?

Microbial ecology has the principal aim of answering these questions by studying the microorganisms and their interactions with each other and the environment through biochemical and molecular methods. Standard culture techniques to characterize microbial ecology, though, involve isolation and characterization of microorganisms using commercial growth media. The major limitation of culture-based techniques is that >99% of the microorganisms in any environment observed though a microscope are not cultivable by standard culturing techniques. Several improved cultivation methods aim to mimic natural environments in terms of nutrients, oxygen, pH, etc. to maximize the cultivable fraction of microorganisms (Rastogi & Sani, 2011).

2.4.1.1. Methylene blue dye reduction test (MBRT)

Viability is an essential analytical measurement for quantifying cell cultures. One of the categories for quantifying cell cultures is based on microscopic examination of stained cells of an organism by using methylene blue, acridine orange or propidium iodide (Bapat, Nandy, Wangikar, & Venkatesh, 2005).

The methylene blue dye reduction method, like the name says, relies on the reduction of the dye by the cells. The methylene blue in a sample containing microorganisms gets reduced to a colorless form at the cell surface via reductase enzymes that exist there. This colorless form of methylene blue is uncharged and lipophilic. Dead cells are unable to reduce the methylene blue and therefore are stained blue (Bapat, Nandy, Wangikar, & Venkatesh, 2005; Kwolek-Mirek & Zadraf-Tecza, 2014).

2.4.1.2. Polymerase Chain Reaction (PCR)

This procedure is widely used to amplify DNA sequences. The PCR is used to amplify a specific fragment of DNA from a complex mixture of starting material, usually called template DNA. It's necessary to know some information about the DNA sequence which flanks the fragment of DNA to be amplified, called target DNA. With this information in mind, two oligonucleotide primers can be synthesized, each of them complementary to a strand of DNA to the 3' side of the target DNA. It might be compared to the DNA replication process, since the outcome is the same: the new DNA strands are based on existing ones.

The PCR have three sets of times and temperatures that are called steps: denaturation, annealing and extension. Each of these steps can be repeated several times and called cycles. In the first cycle, the double-stranded DNA template is denatured by heating the reaction to a temperature above 90ºC. The temperature is then cooled to about 40-60ºC and it highly depends on each PCR system. PCR systems that are not optimized for this step usually give other DNA products in addition to the one target we want to amplify. The annealing step allows the hybridization of the two primers to bind to their complementary sites in the target DNA. The annealed oligonucleotides act as primers for DNA synthesis, since they provide a free 3' hydroxyl group for the DNA polymerase. The extension step is carried out by a thermostable DNA polymerase, usually *Taq* DNA polymerase (Wilson & Walker, 2010).

This synthesis keeps happening for both primers until the new strands are extended along and beyond the target DNA. These new strands, since they extend beyond the target DNA, will have a region near the 3' end that is complementary to the other primer, so they can be used as a template DNA in case of a new DNA synthesis. As the system is taken through several cycles of denaturation, annealing and extension all the new strands act as templates, so the amount of DNA produced will increase exponentially (Wilson & Walker, 2010).

There used to be a problem with PCR reactions because the temperature needed to denature the DNA also denatured the DNA polymerase. This was solved thanks to the existence of a thermostable DNA polymerase, isolated from *Thermus aquaticus* (thermophilic bacteria), found in hot springs. *Taq* DNA polymerase has an optimal temperature of 72ºC and survives high temperatures (up to 96ºC) (Wilson & Walker, 2010).

The PCR specificity relies highly on the design of its oligonucleotide primers. There has to be an extra care while designing them because not only they need to be complementary to the target DNA flanking sequence, but also they must not be complementary between themselves or bind each other to form dimers. They also must be matched in their GC content and have similar annealing temperatures.

This technique has an enormous sensitivity and the samples are easily contaminated. Sometimes all it takes is a bit of DNA from dust particles for the PCR to give misleading results. It's very important to keep the equipment always cleaned (Wilson & Walker, 2010).

2.4.1.3. Electrophoresis

Electrophoresis in agarose or polyacrylamide gels is a technique used to separate DNA molecules (or other macromolecules, like RNA and proteins) according to their size. This technique can be used analytically and can be either qualitative or quantitative. The gels are mainly used using agarose, which comes as dry powdered flakes, and when they are heated with a buffer and allowed to cool, they form a gel. This gel, at a molecular level, is a matrix of agarose molecules that are held together by hydrogen bonds and form small pores. Agarose gels are usually used to separate molecules larger than 100 base pairs while polyacrylamide gels are mainly used for shorter DNA molecules (Academy, n.d.; Wilson & Walker, 2010).

This technique is commonly done with horizontal agarose gels which are placed inside a gel box. This gel box has two poles, one negative and one positive, where a power supply is connected. The agarose gel is placed inside the gel box which is filled with a salt-containing buffer solution that can conduct electrical current. The samples are placed inside small wells that are close to negative pole of the box. All the samples, before going into the wells, are stained with ethidium bromide. This chemical compound is a dye that binds to DNA by inserting itself between base pairs and showcases a red fluorescence when illuminated with ultraviolet light (Academy, n.d.; Wilson & Walker, 2010).

The movement of the DNA molecules within the gel is caused by the current that is applied through the gel. Since the DNA molecules have a negative charge because of the phosphate groups in their sugar-phosphate backbone, they start moving through the gel towards the positive pole. As the gel runs, shorter pieces of DNA travel through the pores of the gel faster than longer ones (Academy, n.d.) (Wilson & Walker, 2010).

2.4.2. Methods for identification of non-cultivable species

The vast majority of microbial communities have yet to be cultured in laboratory. Therefore, the primary source of information relies on their biomolecules such as nucleic acids, lipids and proteins. Culture-independent nucleic acid approaches include analysis of whole genomes or selected genes (such as 16S and 18SRNA for prokaryotes and eukaryotes, respectively). Based on comparative analysis of these rRNA signatures, cellular life has been classified into three primary domains: one eukaryotic (Eukarya) and two prokaryotic (Bacteria and Archaea). These methods have been classified in partial community analysis, like DNA microarrays or Microbial lipid analysis, and whole community analysis, like whole genome sequencing or proteogenomics (Rastogi & Sani, 2011).

2.5. Methods for color evaluation

Color is one of the most relevant parameters for selecting a food. Together with the aspect of the surface of the food product, color is critical for the consumer's acceptance of the product even before it enters the mouth. However, the visual perception of color varies significantly from person to person and according to the environmental conditions.

The determination of color can be carried out by visual inspection or by using a color measuring instrument. In order to establish a more standard pattern for color, results from visual perception can be transformed into numbers and quantified by using a colorimeter and CIELab color space system. Within this system, the color of a sample is given by the distance between the points defining 3 different parameters, the luminosity L* parameter (from white to black) that ranges from 0 to 100, a* parameter (from green to red) and b* parameter (from blue to yellow) both ranging from -120 to 120 (figure 2.3) (León, Mery, Pedreschi, & León, 2006).

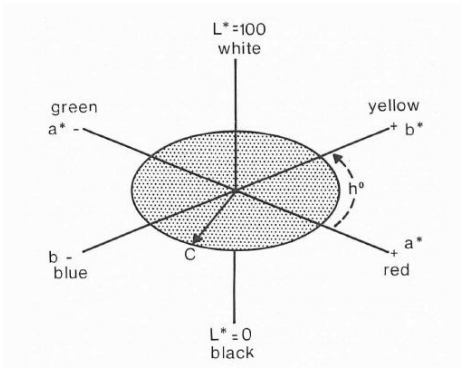

Figure 2.3 - CIELAB color space (source: Weatherall, 1992)

The L*a*b* space is perceptually uniform, which means that the Euclidean distance between two different colors corresponds approximately to the color difference perceived by the human eye. To be able to carry out a detailed characterization of the image of a food item and evaluate more precisely its quality, it is necessary to know the color value of each pixel on its surface. This kind of precise evaluation is hard on heterogenous materials such as food materials (León, Mery, Pedreschi, & León, 2006).

Using this system, we can then compare samples based on the ΔE value calculated according to the following equation: (Weatherall, 1992)

$$\Delta E^*_{ab} = \sqrt{(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2}$$

where

ΔL* means the difference between the value of L parameter determined at time zero and at the time of each sample collection.

Δa* means the difference between the value of “a” parameter determined at time zero and at the time of each sample collection.

Δb* means the difference between the value of “b” parameter determined at time zero and at the time of each sample collection.

2.6. Evaluation of shelf-time and preservation capacity

2.6.1. Methods of evaluation of shelf-life time

Foods are perishable by nature. Several changes can happen in foods during processing and storage. While the food product is in storage, one or more qualities attributes may reach an undesirable state and when this happens the food product is said to have reached the end of its shelf-life. During storage and distribution, foods are exposed to a wide range of environmental conditions, like humidity, oxygen or temperature, that lead to food degradation. As a consequence, the food product may be rejected by the consumer or become harmful to whoever is consuming it. It is imperative that to understand the different reactions that cause food deterioration before evaluation of the shelf-life of foods (Jones, 1994).

Shelf-life is defined as the time between the production and packaging of the product and the point at which it becomes unacceptable under defined environmental conditions. A common practice used to evaluate the shelf-life of a given food product is to determine changes in selected quality characteristics over a period of time. Empirical or analytical techniques may be used to quantify the quality and attributes of food. Enumeration of microorganisms or determination of chemical components of a product are analytical techniques, whereas the use of human subjects to monitor changes of the quality of the product constitute empirical techniques (Jones, 1994).

2.6.2. Challenge tests

A challenge test (can also be named preservative effectiveness test or antimicrobial effectiveness test) is a procedure used to determine if a product (cosmetic or food) is adequately preserved to prevent contamination from raw products or consumer use.

The chemical, physical and microbial properties are important to determine if a preservative is important, but they do not provide enough information if the product will be rightfully preserved. There are other factors that are important like the possible interactions with the containers where the product is stored in (Jones, 1994; IFT, 2003).

Principles to design a challenge test

The adequate level of preservation of the product used during the test needs to be maintained during its shelf-life time, including its use by the consumer. During its usage, the product can be contaminated by several microorganisms, so challenge tests are mostly conducted during the beginning and at the end of the product shelf-life. A suitable challenge test involves the

addition of several bacteria and/or moulds to ensure that microbial inactivation occurs. When conducting a microbiological challenge test, there are some factors that need to be accounted:

1. The selection of appropriate pathogens:
2. The level of challenge inoculum;
3. Preparation and method of inoculation of the inoculum;
4. The duration of the study;
5. Storage conditions;
6. Sample analysis.

The selection of appropriate pathogens

The selection of these pathogens depends highly on the knowledge of the food formulation and history of the food. The ideal organisms for challenge testing are those that have been previously isolated from similar formulations. Additionally, pathogens from previous foodborne outbreaks can be added to check if the formulation is robust enough to inhibit them. Some of those organisms are shown on the table below:

Table 2.2 - Pathogens that may be considered for microbial challenge tests for some food products.

Food type	Type of organism
Salad dressings	Salmonellae and *Staphylococcus aureus*
Dairy products	Salmonellae, *Staphylococcus aureus*, *Clostridium botulinum*, enterohemorrhagic *Escherichia coli*, *Listeria monocytogenes*
Sauces stored at ambient temperature	Salmonellae and *Staphylococcus aureus*
Modified atmosphere packaged products (like meat, poultry or fish)	*Clostridium botulinum* (proteolytic and nonproteolytic strains), enterohemorrhagic *Escherichia coli*, *Listeria monocytogenes*

Source: Adapted from IFT, 2003.

The level of challenge inoculum

The inoculum level used in microbiological challenge studies highly depends on the objective of the study, if it's to determine the product's stability and shelf life or to validate a step in the process to reduce the number of microorganisms. Usually, the inoculum level used to see the microbiological stability of a formulation is 10^2 and 10^3 cells per gram of product. In some products it can be used a higher level of inoculum. If these numbers are too low, there a chance that an incorrect assumption can be about the product stability. Conversely, when a high amount is used,

the preservation system can be overwhelmed by this inappropriate inoculum size and an incorrect conclusion can be assessed. When validating a lethality process, such as heat processing or high-pressure processing, it is necessary an inoculum level of 10^6 and 10^7 cells per gram of product (Jones, 1994; Russel, 2003).

Preparation and method of inoculation of the inoculum

The preparation of the inoculum is an important part of any microbiological challenge testing. The challenge cultures should be grown in media and under conditions suitable for optimal growth of the specific microorganism used. For example, bacterial spores can be stored in water under refrigeration or glycerol in frozen environments (spore suspensions should be diluted in sterile water and immediately heated prior to inoculation). Quantitative counts on the challenge suspensions may be conducted so it's possible to learn the necessary dilutions needed in the challenge product. Enough product needs to be inoculated so that a minimum of two replicates can be done throughout the challenge study (Jones, 1994; Russel, 2003).

The duration of the study

The duration of the study highly depends of the product's shelf-life. It's also advisable to take into consideration the temperature of the product storage. Refrigerated products may be challenged under the target storage temperature, but under abuse temperatures they are held for shorter time

It is also advantageous to test the product beyond its shelf-life because sublethal injury may occur in some products. This can lead to a long lag period, where it's not possible to inoculate the inoculum, but over time, some injured cells might be able to recuperate and grow in the product. This is called the "phoenix" phenomenon and it has been observed in some products. If the product is not tested during its shelf life, there is a high chance you can miss out on the recovery/growth of the challenge organism later (Jones, 1994; Russel, 2003).

The frequency of the testing is determined by the duration of the challenge test. It is advisable to have between 5 and 7 data points over the shelf-life so it's possible to have a grasp of the inoculum behavior. The shelf-life is measured in days, and the recommendation is for the frequency of testing to be daily or multiple times a day. All studies should start with "zero time", that is, analyzing the product right after the inoculation.

Storage conditions

It is very important to understand the range of key factors that control the microbiological stability. There are intrinsic factors that need to be accounted, like the pH value or the water activity, in order to prevent the growth of pathogens or spoilage that would influence the safety of the product during its shelf life (Jones, 1994; Russel, 2003).

It is also important that the test samples are stored in the same kind of packaging as intended for the commercial marketplace. The storage temperature also needs to be within the range at which the product is to be held and distributed.

Sample analysis

Normally, in a microbiological challenge test study, the number of live challenge microorganisms are enumerated at each sample point. It is advisable to have at least a duplicate sample, or a triplicate, for analysis at each point in time. The selection of enumeration media and method (for example direct plating) is very dependent on the type of pathogens used in the study. If the product doesn't possess substantial background microflora, nonselective media can be used.

It is also advisable to analyze the product, including non-inoculated control samples, at each sampling point in the study so we can able to see how the background microflora is behaving during the product's shelf-life (a product with high background microflora may stop the growth of the challenge inoculum) (Jones, 1994; Russel, 2003).

It's very important to track the physicochemical parameters of the product over its shelf-life to see how they change and affect the behavior of the pathogens inoculated. Factors like the pH, water activity, moisture, salt level and/or other variables need to be studied in order to assess the microbiological stability of the product (Jones, 1994; Russel, 2003).

3. Methods

3

3.1. Strains and maintenance media and viability

3.1.1. Strains and culture maintenance

The yeast strains used in this work were *Candida versatilis* ISA2060 obtained from Yeast ISA Culture Collection. *Zygosaccharomyces rouxii* CBS732 and *Aspergillus oryzae* CBS817.72 were obtained from CBS culture collection (Centraalbureau voor Schimmelcultures, The Netherlands). *Zygosaccharomyces rouxii* and *Aspergillus oryzae* were routinely kept in YPD solid media (2% (w/v) glucose, 1% (w/v) bacto peptone, 1% (w/v) yeast extract solidified with 2% (w/v) agar). *Candida versatilis* was kept in solid YPD with 10% (w/v) NaCl.

For challenge tests, the bacteria strains used were: *Escherichia coli* BISA3967, *Salmonella enterica Typhimurium* BISA3969, *Bacillus cereus* BISA4043, Listeria *innocua* BISA3001 and *Staphylococcus aureus* BISA3966, from Bacteria ISA Culture Collection. All the 5 bacteria strains described before were routinely kept in TSA (Triptone soya agar) media at room temperature.

3.2. Production of starters

3.2.1. Miso starter – *koji* preparation

Firstly, the rice was steamed for 30 minutes at a temperature of 100ºC. Once the rice is cooled at room temperature, it was spread in containers together over 3 layers of gauze. After all the rice is spread in the container, "*koji* starter" was sprinkled all over it. After everything is sprinkled, it was covered with gauze. It was left fermenting for two to three and stirred in regular intervals (morning and late in the afternoon).

3.2.2. Yeast starter

Zygosaccharomyces rouxii and *Candida versatilis* starters were prepared from suspensions of these yeasts. Yeasts were grown in YPD and YPD with 10%NaCl, respectively. Suspensions were incubated in an orbital shaker (180rpm) at 28ºC until a dense cell suspension was obtained.

The total number of cells and the number of viable cells were determined by counting using a hemocytometer and methylene blue staining according to the method described in 2.4.1.1.

After counting, cells were centrifuged for 3 minutes (15000 *xg*, 4ºC) and resuspended in water in order to obtain 1x10^6 cells/mL. In the case of starters containing both *Z. rouxii* and *C. versatilis*, suspension was prepared with 0.5x10^6 cell/mL of each yeast strain.

3.3. Media preparation

3.3.1. Cycloheximide preparation

To prepare a concentrated solution with cycloheximide, 250 mg of cycloheximide were weighed and added to a 25 mL volumetric flask. The solution was sterilized by filtration (0.22 µm of pore). The solution was split into 1.5 mL eppendorf tubes and stored in the freezer (-20ºC).

3.3.2. MRS (De Man, Rogosa and Sharpe agar) media

MRS medium was prepared according to the bottle's instructions were followed and 70.3 g were weighed to prepare 1 L of media, this mixture was heated to boiling so that the agar could be dissolved. After the agar is dissolved, the media was split between two 500 mL bottles and both were put inside the autoclave to be sterilized. In the case of MRS with 10% (w/v) NaCl media, NaCl was added to the MRS media, completely dissolved and sterilized the same way. Both media were poured into sterile Petri dishes (around 15 mL per plate).

After the sterilization is complete, the bottles were cooled at room temperature and 500 µL of cycloheximide was added to each of them in order to obtain a final concentration of 10 ppm.

3.3.3. YPD (Yeast extract peptone dextrose) and YPD with 10% (w/v) of NaCl

To prepare this media 1% (w/v) peptone, 2% (w/v) glucose, 0,5% (w/v) yeast extract, 2% (w/v) agar were weighed. This mixture was heated to boiling so that the agar could be dissolved. After the agar was dissolved, the media was split between two 500 mL bottles and both were put inside the autoclave to be sterilized. After the sterilization is complete, the bottles were removed from the autoclave and cooled at room temperature. In the case of YPD with 10% (w/v) NaCl, 100g of NaCl per liter of final media was added and solubilized before sterilization. Both media were poured into sterile Petri dishes (around 15 mL per plate).

3.3.4. TSA (Tryptone Soya Agar)

TSA media was prepared according to the manufacturer instructions. To prepare this media 30g of TSB and 20g of agar were weighed in order to make 1 L of media. This mixture was heated to boiling so that the agar could be dissolved, and then sterilized by autoclaving. The bottles were cooled at room temperature and plated.

3.3.5. *Bacillus cereus* agar media

To prepare Bacillus cereus media, 44.5 g of dehydrated media was weighed and dissolved in 900 mL of distilled water by stirring. It was stirred constantly until complete dissolution. Once complete dissolution is achieved, it was sterilized on the autoclave. After the sterilization is complete, it was cooled down and maintained in a molten state at about 45ºC. Once it's cooled enough, 100 mL of the sterile egg yolk emulsion with Polymyxin B were added and homogenized by hand stirring, and plated.

3.3.6. Compass ECC agar media

To prepare Compass ECC agar, 40.8 g of dehydrated media was weighed and dissolved in 1 L of distilled water. It was stirred constantly until complete dissolution. Once complete dissolution is achieved, it was sterilized on the autoclave. After the sterilization is complete, it was cooled down and maintained in a molten state at about 45ºC until plate preparation.

3.3.7. PALCAM agar media

To prepare PALCAM agar media, it was weighed 68.9 g of dehydrated media and dissolved in 1 L of distilled water. It was stirred constantly until complete dissolution. Once complete dissolution is achieved, it was sterilized on the autoclave. After the sterilization is complete, it was maintained in a molten state at about 45ºC. Once it's cooled enough, 10 mL of rehydrated supplement BS004 Qsp 500 mL (5 mL sterile water added) were added and homogeneized by hand stirring and plated.

3.3.8. XLD agar media

To prepare XLD media, 58.0 g of dehydrated media was weighed and dissolved in 1 L of distilled water. The mixture was slowly brought to boiling while being constantly stirred until complete dissolution. It was maintained in a molten state at about 45ºC and plated.

3.3.9. Baird parker agar media preparation.

To prepare Baird Parker media, 58.0 g of dehydrated media was weighed and dissolved in 950 mL of distilled water. Once complete dissolution is achieved, it was sterilized on the autoclave. After the sterilization is complete, it was maintained in a molten state at about 45ºC. Previously to plate preparation, 50 mL of the sterile egg yolk tellurite enrichment were added and homogenized by hand stirring. The plates were plated and left to solidify on a flat surface.

3.4. Production of miso

Miso paste from grass pea and from soybean was prepared according to the following formulation:

- 57.9% of soybean and grass pea;
- 29% of *koji*;
- 10% salt;
- 3.1% of yeast starter in water ($1.0x10^6$ cells per miso gram).

Firstly, both the soybeans and grass pea were left in water overnight. In the next day, both were cooked under positive pressure for 20 minutes (1 atm). After they were cooled, they were grinded and saved for later.

Once both the soybeans and grass pea were grounded, all the ingredients on the previous list were mixed together, and the mixture was homogenized. They were then split into small sterilized containers with salt rubbed on its walls. After the mixture is tight inside the container, another layer of salt was added to the top, and the container was closed. These containers were then left to ferment for some months.

3.5. Evaluation of the color

Color evaluation was performed using a colorimeter MINOLTA (Model CR-300, Japan), with standard light source D65. L*, a* and b* parameters were obtained as the mean of 8 different measurements. calibration was made with against a standard white pattern.

3.6. Miso's microbiota evaluation

3.6.1. Macroscopic and microscopic analysis

When the plates exhibited full grown colonies, they were counted based on the aspect of each of them. These colonies were then selected and observed under the microscope to divide these types of colonies in three categories: moulds, yeasts and bacteria.

3.6.2. Determination of viable culturable cell number

In each different month, a 1 g sample from each type of miso it was collected. Decimal series of the samples (from 10^{-1} to 10^{-4}) were prepared using sterile distilled water. 100 µL of the miso sample was inoculated on each plate (3 plates for each dilution). On the MRS media dillutions 10^{-1} to the 10^{-3} dilution were inoculated and on the YPD media from the 10^{-1} dilution to the 10^{-4} dilution were inoculated. The inoculated plates were incubated at room temperature (≈25ºC).

3.6.3. Yeasts' viability determination

A 1:10 (v/v) dilution was made with the *C. versatilis or Z. rouxii* suspension (100 µL) and sterile miliQ water (900 µL). Cells were stained for 15 minutes with methylene blue, a 1:2 (v/v) dilution was made. After incubation, cells were counted under the microscope using a hemocytometer.

3.6.4. Identification of the microorganisms by molecular methods

3.6.4.1. DNA extraction

In the microflow laminar workstation, cells from isolated colonies were removed from the plates with the help of a sterile toothpick and inoculated into 5 mL of media and incubated at 28 ºC. Once they were grown, 4 mL (2x2 mL) were poured into eppendorf tubes, centrifuged (16000 rpm for 1 minute) and the supernatant was discarded. The pellet was washed with sterile water.

In order to break the cells, 100 µL of the 10% SDS solution was added to each eppendorf and, with the help of the vortex, they were mixed until complete resuspension. Meanwhile, the water bath was heated. Once the water bath is at the required temperature, the eppendorfs were incubated in a water bath at 90ºC for 10 minutes. After, the samples were centrifuged for 10 minutes at 16000 rpm. The supernatant obtained was kept, transferred to another eppendorf and stored at -20ºC.

3.6.4.2. Polymerase Chain Reaction

The set of primers chosen, and their sequences are shown on table 3.1 and 3.2:

Table 3.1 - Primer sequences for yeasts identification by polymerase chain reaction and sequencing.

Primer	Primer sequence
ITS1	5´TCC GTA GGT GAA CCT TGC GG 3´
ITS4	5´TCC TCC GCT TAT TGA TAT GC 3´

Table 3.2 - Primer sequences for identification by polymerase chain reaction and sequencing.

Primer	Primer sequence
sara-pA	5' AGA GTT TGA TCC TGG CTC AG 3'
sara-pH	5' AAG GAGGTG ATC CAG CCG 3'

A master mix for PCR reaction was prepared according to Table 3.3.

Table 3.3 – Polymerase chain reaction master mix

Solution	Quantity
10x reaction buffer	**5** µL
H_2O MilliQ	**40,8** µL
ITS1 or Sara-pA primer	**1** µL
ITS4 or Sara-pB primer	**1** µL
dNTPs	**1** µL
NZYLong DNA polymerase	**0,2** µL

1 µL of DNA samples was added to 49 µL of the master mix in 100 µL PCR tubes. These tubes were put on the Polymerase chain reaction machine and the PCR program presented in table 3.4 (for yeasts) and 3.5 (for bacteria) was run:

Table 3.4 – Polymerase chain reaction program for yeasts

Temperature (ºC)	Time	Number of cycles
95	**10 minutes**	**1**
95	**1 minute**	**30**
49	**1 minute**	
72	**1 minute**	
72	**7 minutes**	**1**
4	**indefinite**	**1**

Table 3.5 – Polymerase chain reaction program for bacteria

Temperature (ºC)	Time	Number of cycles
94	**5 minutes**	**34**
94	**30 seconds**	
56	**30 seconds**	
72	**1 minute**	
72	**5 minutes**	**1**
4	**indefinite**	**1**

3.6.4.3. Purification using the Wizard Column method

After the polymerase chain reaction, DNA was purified with Wizard columns, according to the manufacturer instructions. Briefly, 50 µL of membrane binding solution (provided by the kit) was added. The mixture was then transferred to one SV minicolumn inserted into the collection tube and incubated for 1 minute at room temperature. After, the columns were centrifuged for 1 minute at 16000 *xg*. The flowthrough was discarded and the minicolumn was inserted again into the collection tube. DNA was washed twice by adding 700 µL and 500 µL of membrane wash solution and centrifuged for 1 minute at 16000 *xg* each time. The flowthrough was discarded and the tubes were centrifuged for 1 minute at 16000 *xg* so the ethanol could evaporate completely.

The minicolumn was carefully transferred to a 1.5 mL eppendorff tube and 50 µL of nuclease-free water was added to the minicolumn, incubated at room temperature for 1 minute and centrifuged for 1 minute at 16000 *xg*. The minicolumn was then discarded and the sample was transferred to a new 1.5 mL eppendorf and stored at -20ºC.

3.6.4.4. Electrophoresis

The electrophoresis gel contained 0.8% of agarose and then 50 mL of TAE buffer (Tris base, acetic acid and EDTA) was added and the solution was prepared by heating until all agarose was dissolved. The gel was cooled, 2 µL of gel red stain was added, and the gel was put on the mold. When the matrix is completely dried, it was removed from the mold and put on the electrophoresis apparatus containing TAE buffer (Tris base, acetic acid and EDTA).

For sample preparation, 10 µL of the DNA extraction samples were taken and 2 µl of loading dye was added. The size marker used was 5 µL λDNA HindIII with 2 µL of loading dye. The samples were loaded in the gel and electrophoresis was run by applying an electrical field (60V for 60 minutes).

In the case of the purified DNA samples, the same method was performed, but using 1.5% (w/v) agarose and preparing samples with 2 µL of the DNA sample plus 8 µL of sterile water and 2 µL of loading dye. All of the gels were visualized with the help of an UV light machine.

3.6.4.5. Sequencing of the ITS1-ITS4 region of the rDNA of yeasts and the rDNA of acid lactic bacteria.

After purification, the samples were left to concentrate in a vacuum desiccator with vacuum for 1 day. 10 µL of sterile water was added to resuspend the dried DNA. These samples were then sent to StabVida, Lda. and automated sequenced.

3.6.4.6. Identification using the BLAST database

The nucleotide sequence provided by StabVida, Lda. (Caparica, Portugal) was introduced in the BLAST database (available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi) to be identified.

A Blastn search was performed against yeast and bacteria sequences available, using the parameters defined by default.

3.7. Evaluation of the self-preservation capacity of miso by challenge tests

3.7.1. Pathogenic cell suspensions preparation

Inside the microflow laminar workstation and with the help of a sterile toothpick, the colonies were picked, resuspended 5 mL of TSB media and incubated at 30ºC until visible strong growth was observed.

Decimal serial dilutions of the suspension of each pathogenic (from 10^{-1} up to 10^{-6}) were prepared. After, 100 µL of each dilution was inoculated on each plate in triplicate.

3.7.2. Calibration curves

Calibration curves for establish the correlation between cell number and OD at 560nm were performed by measuring OD of serial dilution of a suspension with known CFU/mL titer.

To make the *Bacillus cereus* calibration curve shown below, several dilutions were made until the sample had a value of 0.500 of absorbance measured at 560 nm. Once the sample has a value close to 0.05 of absorbance, the dilutions were stopped.

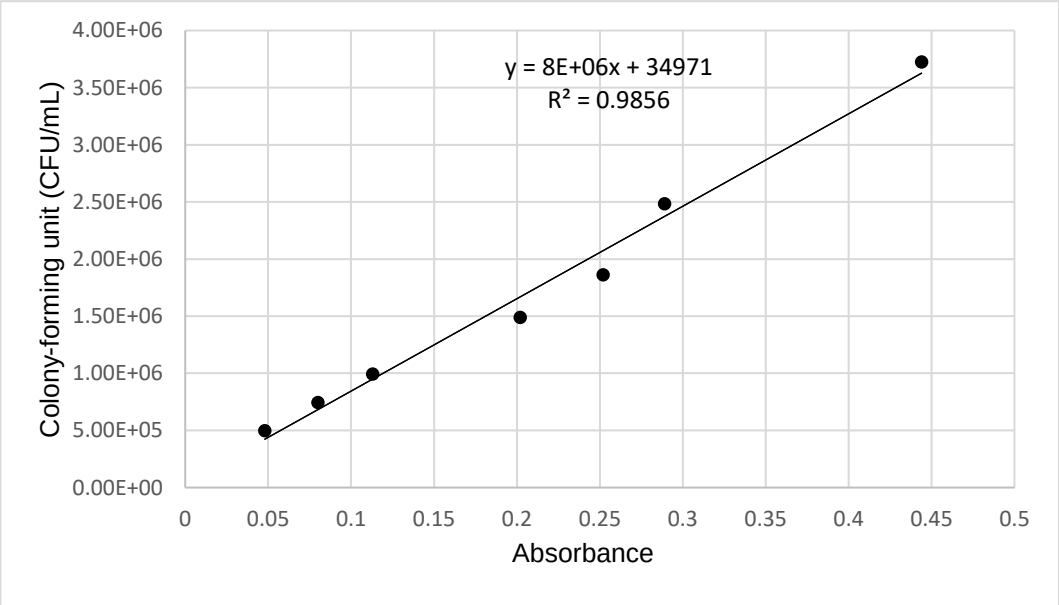

Figure 3.1 – Graphic representation of the calibration curve for *Bacillus cereus*.

To make the *Escherichia coli* calibration curve shown below, several dilutions were made until the sample had a value of 0.500 of absorbance measured at 560 nm. Once the sample has a value close to 0.05 of absorbance, the dilutions were stopped.

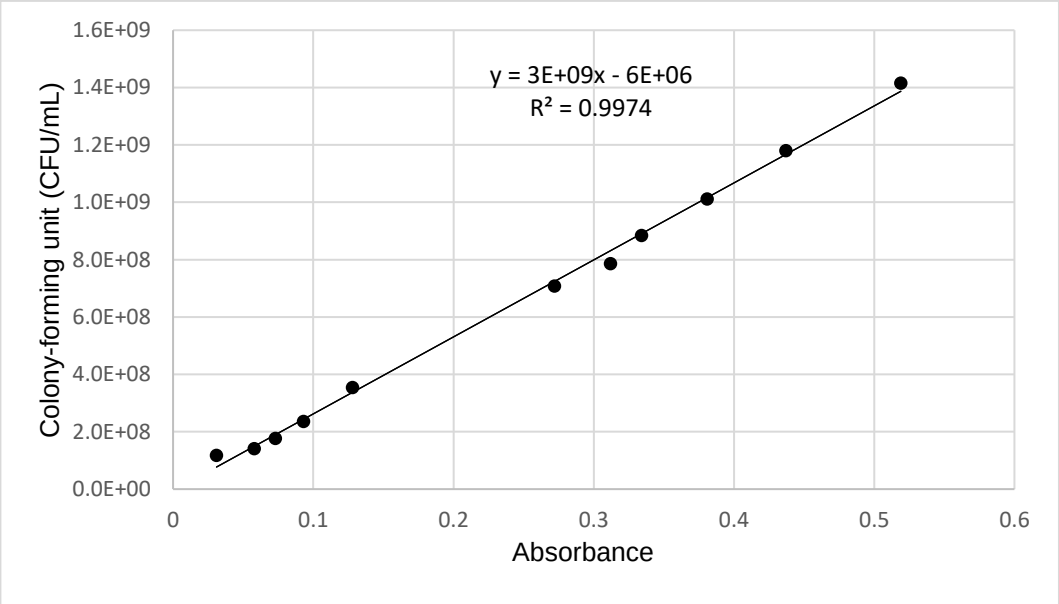

Figure 3.2 - Graphic representation of the calibration curve for *Escherichia coli*.

To make the *Listeria innocua* calibration curve shown below, several dilutions were made until the sample had a value of 0.500 of absorbance measured at 600 nm. Once the sample has a value close to 0.05 of absorbance, the dilutions were stopped.

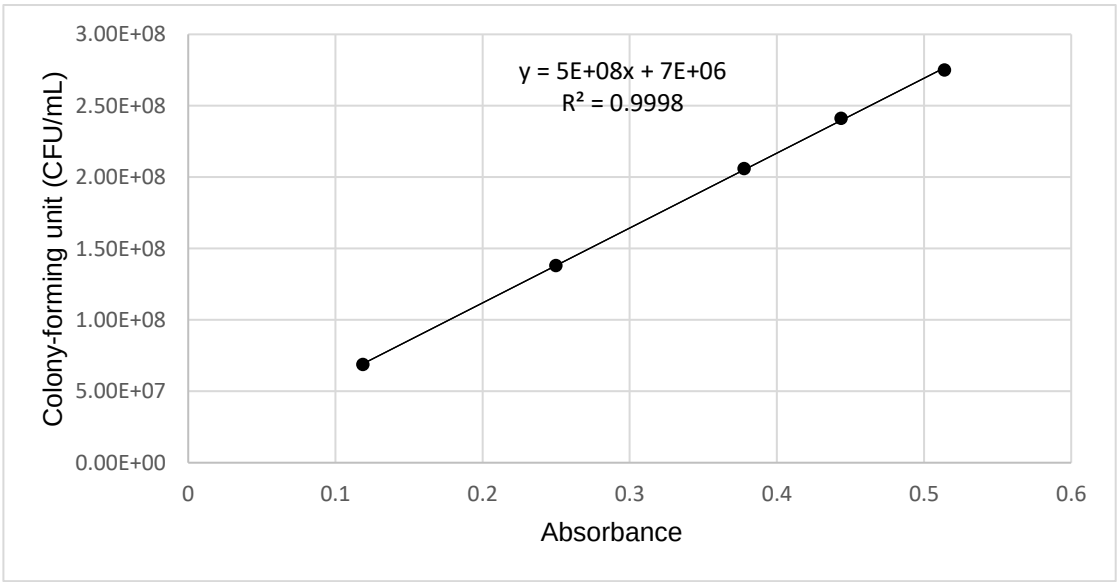

Figure 3.3 - Graphic representation of the calibration curve for *Listeria innocua*.

To make the *Salmonella enterica Typhimurium* calibration curve shown below, several dilutions were made until the sample had a value of 0.500 of absorbance measured at 560 nm. Once the sample has a value close to 0.05 of absorbance, the dilutions were stopped.

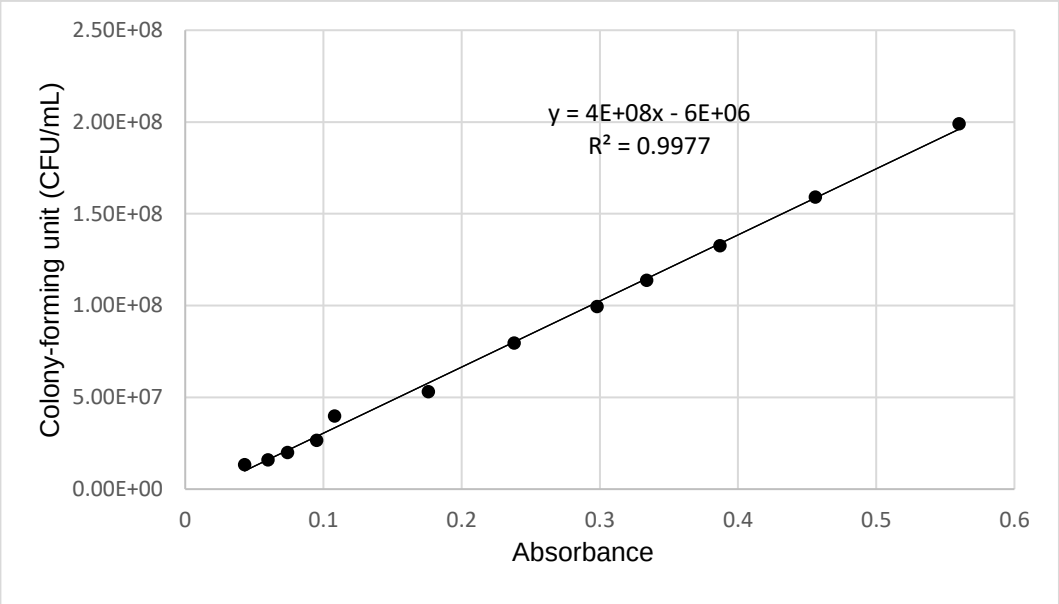

Figure 3.4 - Graphic representation of the calibration curve for *Salmonella enterica Typhimurium*.

To make the *Staphylococcus aureus* calibration curve shown below, several dilutions were made until the sample had a value of 0.500 of absorbance measured at 560 nm. Once the sample has a value close to 0.05 of absorbance, the dilutions were stopped.

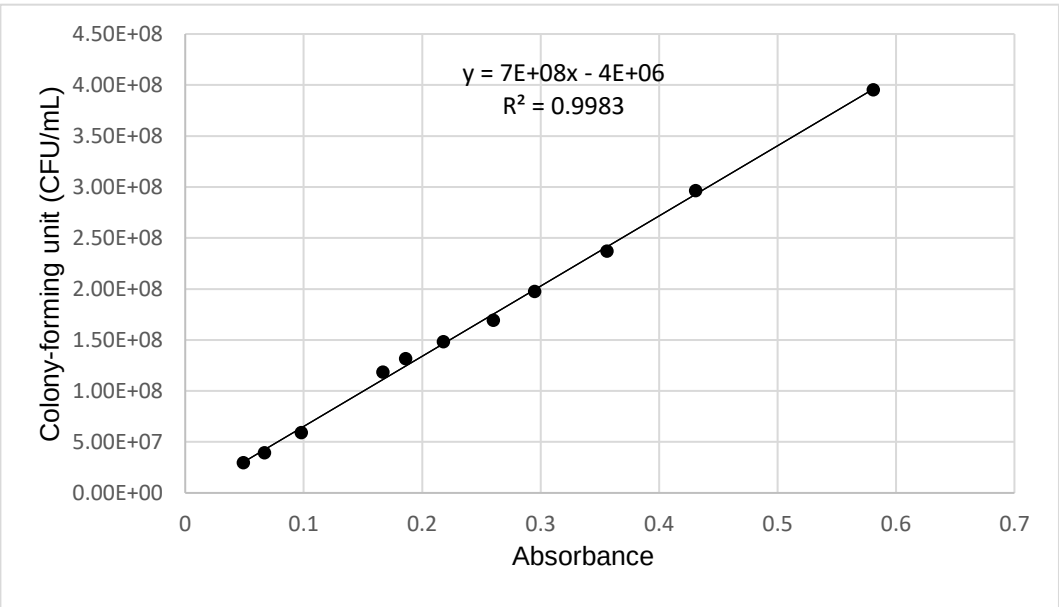

Figure 3.5 – Graphic representation of the calibration curve for *Staphylococcus aureus*.

3.7.3. Miso preparation and inoculation

Each sample of grass pea miso was firstly sterilized. Once the sterilization was complete, the samples were cooled at room temperature. After the samples are cooled, 20g of miso was weighed and separated into 3 different sterile jars.

With the help of the calibration curve, the amount of each pathogenic needed to be added to the miso was calculated in order to obtain $1x10^6$ cells per gram of miso and resuspended in 6 mL of water.

In each different jar it was added 2 mL of the previously prepared pathogenic solution. These jars were then stored in the fridge (4ºC), at room temperature (≈25ºC) and in the oven (37ºC).

3.7.4. Sampling and plating

Right after the grass pea miso was inoculated with the pathogenic solution, a sample was collected and inoculated in all 5 different specific media. The following samples were collected after 2 days, 4 days, 7 days, 14 days, 30 days and 60 days.

It was collected 2 g of each grass pea miso sample at different temperatures. It was added enough water to concentrate our sample. Several dilutions were made after (from 10^{-1} to 10^{-6}) and 100 µL of the miso sample was inoculated on each plate in duplicate. All the plates were then incubated at 37ºC except *Bacillus cereus* agar plates which were incubated at 28ºC.

4. Results and discussion

4

4.1. Starter's viability for the inoculation of miso

4.1.1.1. *Candida versatilis*

To assess the viability of *Candida versatilis*, the methylene blue staining method (explained on section 2.4.1.1) was used. With the help of a hemocytometer and a microscope, 5 pictures were taken and used to see the viability of this yeast.

In the five pictures taken, it was possible to count an average of twenty-five viable cells. After all the cells in each picture were counted, calculations were made in order to assess the number of viable cells we would be introducing in our miso samples – $1.3x10^6$ viable cells per g of miso.

4.1.1.2. *Zygosaccharomyces rouxii*

To assess the viability of *Zygosaccharomyces rouxii*, the methylene blue staining method (explained on section 2.4.1.1) was used. With the help of a hemocytometer and a microscope, 5 pictures were taken and used to see the viability of this yeast.

In the five pictures taken, it was possible to count an average of seventeen viable cells. After all the cells in each picture were counted, calculations were made in order to assess the number of viable cells we would be introducing in our miso samples – $1x10^6$ viable cells per g of miso.

4.2. Evolution of miso's microbiota and maturation

4.2.1. Color evolution perceived by visual observation.

By simply looking at the samples throughout time, it was possible to see the difference in their aspect among the same type of miso. Figures 4.1 and 4.2 show the evolution of the color of miso during 6 months of fermentation.

Figure 4.1 - Evolution of the color of soybean miso using the traditional recipe (left) and with the addition of starters (right).

The differences between the soybean miso using the traditional recipe and adding starters are very visible in figure 4.1. It's possible to see that the evolution of the color in the traditional recipe is way more pronounced, starting with a more green/brown color and ending up with a black color while the soybean miso with starters maintains the same color scheme throughout time starting on a light brown color and ending up 6 months later with a dark brown color. The slower evolution on the color scheme of the soybean miso with the addition of starters is due to the fact that the starter culture used is not yet optimized. If this study had been prolonged for more time, the color would eventually be the same as the soybean miso using the traditional recipe (6 months).

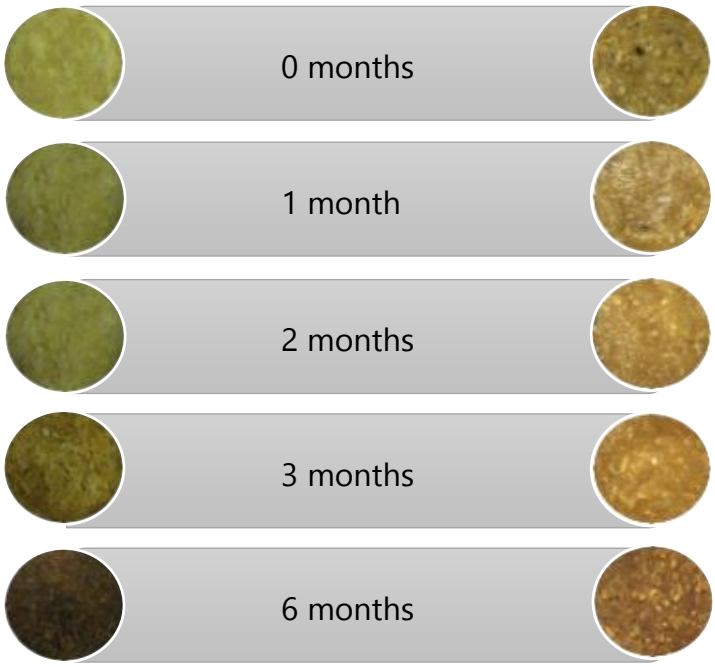

Figure 4.2 - Evolution of the color of grass pea miso using the traditional recipe (left) and with the addition of starters (right).

The differences between the soybean miso using the traditional recipe and adding starters are very visible in figure 4.2. It's possible to see that the evolution of the color in the traditional recipe is more pronounced, starting with a more green/brown color and ending up with a black color while the soybean miso with starters maintains the same color scheme throughout time starting on a light brown color and ending up 6 months later with a dark brown color. The slower evolution on the color scheme of the grass pea miso with the addition of starters is due to the fact that the starter culture used is not yet optimized. If this study had been prolonged for more time, the color would eventually be the same as the grass pea miso using the traditional recipe (6 months).

4.2.2. Color evolution perceived instrumentally.

We also measured color evolution using a colorimeter. With the help of this instrument, it was possible to take 3 different parameters (L*, a* and b*) and use it to calculate ΔE* according to the equation described in section 2.5.

Figure 4.3 shows ΔE* values for both types of miso (soybean and grass pea) with the traditional recipe and by using starters.

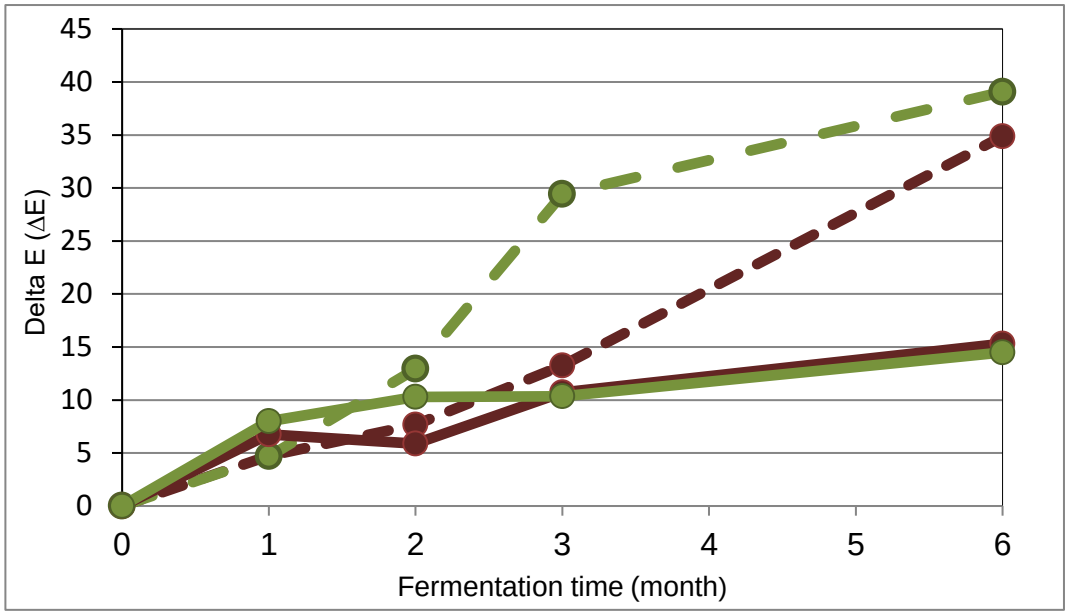

Figure 4.3 - Values of ΔE* for the traditional miso and the miso using starters. The green lines represent soybean miso while the brown lines represent the grass pea miso. The dash lines represent the miso samples made using the traditional recipe while the solid lines represent the addition of starters (*Candida versatilis* and *Zygosaccharomyces rouxii*).

The ΔE is a value that measures the change in visual perception of two given colors. According to figure 4.3, it's possible to see that the traditional recipe of miso has a bigger difference between the ΔE values before it starts fermenting and after 6 months of fermentation. The addition of starters doesn't make the color change noticeable as it can also be seen in figures 4.1 and 4.2.

The same conditions and starter cultures were used for the grass pea miso. Figure 4.4 shows ΔE* values for grass pea miso by using starters.

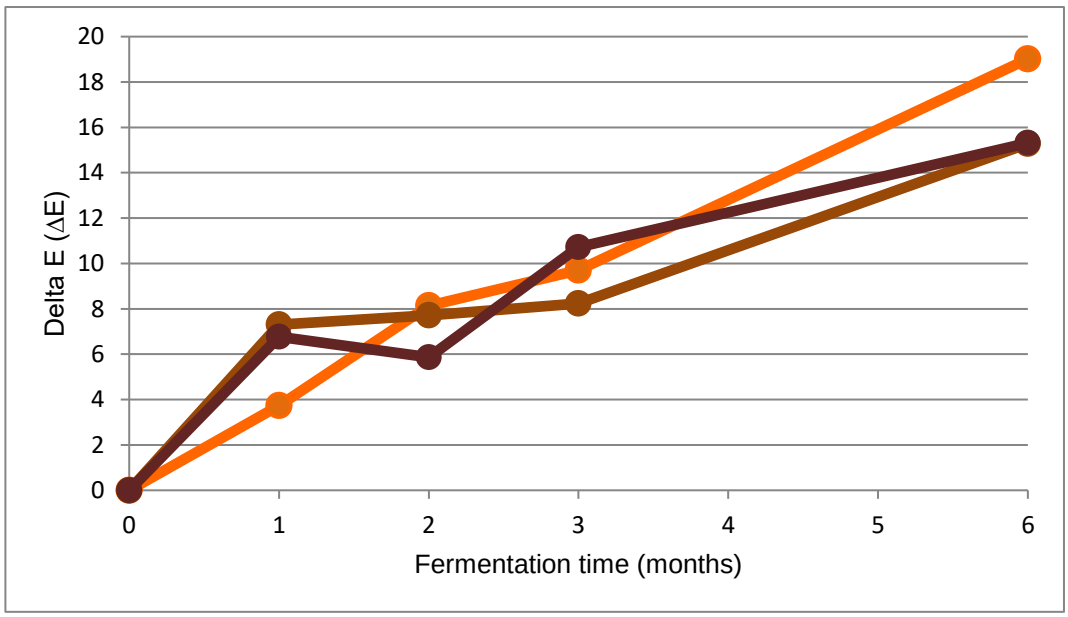

Figure 4.4 - Values of ΔE* for the grass pea miso using starters. The orange line represents the values of ΔE* for the grass pea miso with *Candida versatilis*, the light brown line represents the values of ΔE for the grass pea miso with *Zygosaccharomyces rouxii* and the dark brown line represents the values of ΔE for the grass pea miso with *Candida versatilis* and *Zygosaccharomyces rouxii*.

According to figure 4.4 it's possible to see that difference between the ΔE values among all 3 grass pea miso samples is not much different. The same variations appear on all the 3 samples even if the grass pea miso with *Candida versatilis* and *Zygosaccharomyces rouxii* has a smaller difference between the first sample (0 months) and the third sample (2 months) than the first sample (0 months) and the second sample (1 month).

The same conditions and starter cultures were used for the soybean pea miso. Figure 4.5 shows ΔE* values for soybean miso by using starters.

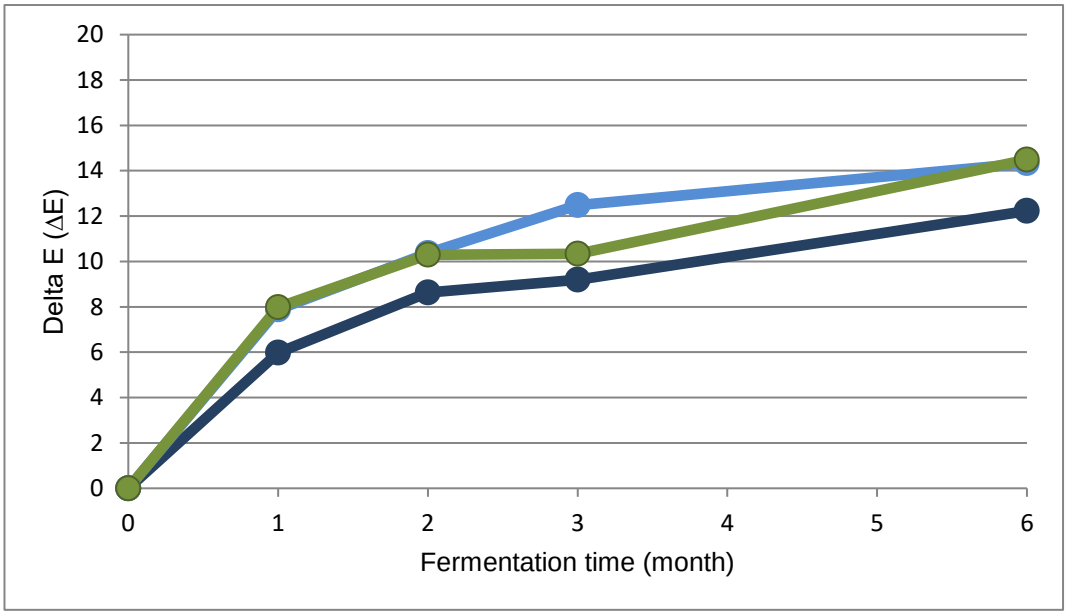

Figure 4.5 - Values of ΔE* for the soybean miso using starters. The dark blue line represents the values of ΔE* for the soybean pea miso with *Candida versatilis*, the light blue line represents the values of ΔE for the soybean pea miso with *Zygosaccharomyces rouxii*.

According to figure 4.5 it's possible to see that difference between the ΔE values among all 3 grass pea miso samples is not much different. The same variations appear on all the 3 samples even if the values of ΔE are slightly different.

4.3. Evaluation of evolution of miso's microbiota

4.3.1. Soybean miso inoculated with *Candida versatilis and Zygosaccharomyces rouxii*

After all the plates were inoculated, these were left at room temperature. When they started showing signs of growth, the colonies were counted. This procedure was made for 8 months (1 sample per month except in the first month where a sample with 15 days was inoculated). Table 4.1 shows the average cell counts (as well as the standard deviation) of the microorganisms present in all the media used after 0, 60 and 180 days of the start of the fermentation process.

Table 4.1 - Average cell counts (as well as the standard deviation) for moulds, yeasts and bacteria in all media used after 0, 60 and 180 days of the start of the fermentation process for soybean miso inoculated with *Candida versatilis* and *Zygosaccharomyces rouxii*.

	Moulds		**Yeasts**		**Bacteria**	
	Mean (CFU/mL)	**Standard deviation (CFU/mL)**	**Mean (CFU/mL)**	**Standard deviation (CFU/mL)**	**Mean (CFU/mL)**	**Standard deviation (CFU/mL)**
0 days	2.21×10^5	4.18×10^4	1.16×10^4	1.02×10^4	1.19×10^5	5.07×10^4
60 days	5.64×10^3	$9{,}15 \times 10^4$	6.67×10^3	0	9.37×10^4	3.31×10^4
180 days	$<1.00 \times 10^3$	0	$<1.00 \times 10^3$	0	7.62×10^4	4.93×10^4

Table 4.1 shows that the mould population cell counts are high in the early stages of maturation but starts to decrease in the 2nd month of fermentation. Even though there are not many studies in the miso's microbiota area, the same variation happens on soy sauce (Xu, et al., 2016) and *doenjang* (Kum, et al., 2015) which are 2 fermented soybean products. The yeast population keeps decreasing throughout the 6 months of fermentation.

The bacterial population decreases throughout the 6 months of microbiota development. The cell counts start high (10^5 CFU/mL) and decrease during the fermentation process to 10^4 CFU/mL after 2 months. This same kind of decrease was reported in a previous study regarding the analysis of bacterial flora in miso fermentation (Onda, Yanagida, Tsuji, Shinohara, & Yokotsuka, 2003).

In the first days of the fermentation process, there were some yellow and transparent bacteria which disappeared after the 2nd month of fermentation.

4.3.2. Soybean miso inoculated with *Zygosaccharomyces rouxii*

After all the plates were inoculated, these were left at room temperature. When they started showing signs of growth, the colonies were counted. This procedure was made for 8 months (1 sample per month except in the first month where a sample with 15 days was inoculated). Table 4.2 shows the average cell counts (as well as the standard deviation) of the microorganisms present in all the media used after 0, 60 and 180 days of the start of the fermentation process.

Table 4.2 - Average cell counts (as well as the standard deviation) for moulds, yeasts and bacteria in all media used after 0, 60 and 180 days of the start of the fermentation process for soybean miso inoculated with *Zygosaccharomyces rouxii*.

	Moulds		**Yeasts**		**Bacteria**	
	Mean (CFU/mL)	**Standard deviation (CFU/mL)**	**Mean (CFU/mL)**	**Standard deviation (CFU/mL)**	**Mean (CFU/mL)**	**Standard deviation (CFU/mL)**
0 days	2.60×10^5	4.78×10^4	$<1.00 \times 10^3$	0	1.86×10^5	1.61×10^5
60 days	5.64×10^3	6.98×10^4	$<1.00 \times 10^3$	0	1.96×10^4	2.02×10^4
180 days	$<1.00 \times 10^3$	0	$<1.00 \times 10^3$	0	2.12×10^4	1.63×10^3

Table 4.2 shows that the mould population cell counts are high in the early stages of maturation but starts to decrease in the 2nd month of fermentation. Even though there are not many studies in the miso's microbiota area, the same variation happens on soy sauce (Xu, et al., 2016) and *doenjang* (Kum, et al., 2015) which are 2 fermented soybean products. There was no growth of the yeast population in this miso.

The bacterial population decreases throughout the 6 months of microbiota development. The cell counts start high (10^5 CFU/mL) and decrease during the fermentation process to 10^4 CFU/mL after 2 months. This same kind of decrease was reported in a previous study regarding the analysis of bacterial flora in miso fermentation (Onda, Yanagida, Tsuji, Shinohara, & Yokotsuka, 2003).

In the first days of the fermentation process, there were some yellow and transparent bacteria which disappeared after the 1st month of fermentation.

4.3.3. Soybean miso inoculated with *Candida versatilis*

After all the plates were inoculated, these were left at room temperature. When they started showing signs of growth, the colonies were counted. This procedure was made for 8 months (1 sample per month except in the first month where a sample with 15 days was inoculated). Table 4.3 shows the average cell counts (as well as the standard deviation) of the microorganisms present in all the media used after 0, 60 and 180 days of the start of the fermentation process.

Table 4.3 - Average cell counts (as well as the standard deviation) for moulds, yeasts and bacteria in all media used after 0, 60 and 180 days of the start of the fermentation process for soybean miso inoculated with *Candida versatilis*.

	Moulds		**Yeasts**		**Bacteria**	
	Mean (CFU/mL)	**Standard deviation (CFU/mL)**	**Mean (CFU/mL)**	**Standard deviation (CFU/mL)**	**Mean (CFU/mL)**	**Standard deviation (CFU/mL)**
0 days	$1.35x10^5$	$5.33x10^4$	$<1.00 x10^3$	0	$2.41x10^5$	$8.41x10^4$
60 days	$2.82x10^3$	$2.57x10^3$	$<1.00 x10^3$	0	$1.85x10^5$	$7.36x10^4$
180 days	$2.75x10^3$	$2.01x10^3$	$<1.00 x10^3$	0	$3.30x10^4$	$4.24x10^2$

Table 4.3 shows that the mould population cell counts are high in the early stages of maturation but starts to decrease in the 2nd month of fermentation. Even though there are not many studies in the miso's microbiota area, the same variation happens on soy sauce (Xu, et al., 2016) and *doenjang* (Kum, et al., 2015) which are 2 fermented soybean products. There was no growth of the yeast population in this miso.

The bacterial population decreases throughout the 6 months of microbiota development. The cell counts start high (10^5 CFU/mL) and decrease during the fermentation process to 10^4 CFU/mL after 2 months. This same kind of decrease was reported in a previous study regarding the analysis of bacterial flora in miso fermentation (Onda, Yanagida, Tsuji, Shinohara, & Yokotsuka, 2003).

In the first days of the fermentation process, there were some yellow and transparent bacteria which disappeared after the 1st month of fermentation.

4.3.4. Grass pea miso with *Candida versatilis and Zygosaccharomyces rouxii*

After all the plates were inoculated, these were left at room temperature. When they started showing signs of growth, the colonies were counted. This procedure was made for 8 months (1 sample per month except in the first month where a sample with 15 days was inoculated). Table 4.4 shows the average cell counts (as well as the standard deviation) of the microorganisms present in all the media used after 0, 60 and 180 days of the start of the fermentation process.

Table 4.4 - Average cell counts (as well as the standard deviation) for moulds, yeasts and bacteria in all media used after 0, 60 and 180 days of the start of the fermentation process for grass pea miso inoculated with *Candida versatilis* and *Zygosaccharomyces rouxii*.

	Moulds		**Yeasts**		**Bacteria**	
	Mean (CFU/mL)	**Standard deviation (CFU/mL)**	**Mean (CFU/mL)**	**Standard deviation (CFU/mL)**	**Mean (CFU/mL)**	**Standard deviation (CFU/mL)**
0 days	$1.12x10^5$	$1.25x10^5$	$1.81x10^4$	$9.00x10^3$	$1.67x10^5$	$1.56x10^5$
60 days	$1.82x10^3$	$2.14x10^3$	$1.93x10^5$	$2.33x10^5$	$2.69x10^5$	$2.47x10^4$
180 days	$<1.00 x10^3$	0	$1.68x10^5$	$1.53x10^5$	$8.00x10^4$	0

Table 4.4 shows that the mould population cell counts are high in the early stages of maturation but starts to decrease in the 2nd month of fermentation. Even though there are not many studies in the miso's microbiota area, the same variation happens on soy sauce (Xu, et al., 2016) and *doenjang* (Kum, et al., 2015) which are 2 fermented soybean products.

The yeast population increases throughout the 6 months of microbiota development. The cell counts start at 10^4 CFU/mL and increase during the fermentation process to 10^5 CFU/mL after 2 months. This same kind of increase was reported in a previous study regarding the analysis of bacterial flora in miso fermentation (Onda, Yanagida, Tsuji, Shinohara, & Yokotsuka, 2003).

The bacterial population decreases throughout the 6 months of microbiota development. The cell counts start high (10^5 CFU/mL) and decrease during the fermentation process to 10^4 CFU/mL after 2 months. This same kind of decrease was reported in a previous study regarding the analysis of bacterial flora in miso fermentation (Onda, Yanagida, Tsuji, Shinohara, & Yokotsuka, 2003).

In the first days of the fermentation process, there were some yellow and transparent bacteria which disappeared after the 2nd month of fermentation.

4.3.5. Grass pea miso with *Zygosaccharomyces rouxii*

After all the plates were inoculated, these were left at room temperature. When they started showing signs of growth, the colonies were counted. This procedure was made for 8 months (1 sample per month except in the first month where a sample with 15 days was inoculated). Table 4.5 shows the average cell counts (as well as the standard deviation) of the microorganisms present in all the media used after 0, 60 and 180 days of the start of the fermentation process.

Table 4.5 - Average cell counts (as well as the standard deviation) for moulds, yeasts and bacteria in all media used after 0, 60 and 180 days of the start of the fermentation process for grass pea miso inoculated with *Zygosaccharomyces rouxii*.

	Moulds		**Yeasts**		**Bacteria**	
	Mean (CFU/mL)	**Standard deviation (CFU/mL)**	**Mean (CFU/mL)**	**Standard deviation (CFU/mL)**	**Mean (CFU/mL)**	**Standard deviation (CFU/mL)**
0 days	2.26×10^5	9.50×10^4	0	0	1.01×10^5	4.62×10^4
60 days	$<1.00 \times 10^3$	0	0	0	$5.07 * 10^4$	1.04×10^4
180 days	$<1.00 \times 10^3$	0	1.54×10^5	2.07×10^5	7.02×10^4	2.12×10^2

Table 4.5 shows that the mould population cell counts are high in the early stages of maturation but starts to decrease in the 2nd month of fermentation. Even though there are not many studies in the miso's microbiota area, the same variation happens on soy sauce (Xu, et al., 2016) and *doenjang* (Kum, et al., 2015) which are 2 fermented soybean products.

In the yeast population cell counts, there are only cell counts (10^5 CFU/mL) after the 6th month of fermentation and in the two following months the cell counts are the same.

The bacterial population decreases throughout the 6 months of microbiota development. The cell counts start high (10^5 CFU/mL) and decrease during the fermentation process to 10^4 CFU/mL after 2 months. This same kind of decrease was reported in a previous study regarding the analysis of bacterial flora in miso fermentation (Onda, Yanagida, Tsuji, Shinohara, & Yokotsuka, 2003).

In the first days of the fermentation process, there were some yellow and transparent bacteria which disappeared after the 1st month of fermentation.

4.3.6. Grass pea miso with *Candida versatilis*

After all the plates were inoculated, these were left at room temperature. When they started showing signs of growth, the colonies were counted. This procedure was made for 8 months (1 sample per month except in the first month where a sample with 15 days was inoculated). Table 4.6 shows the average cell counts (as well as the standard deviation) of the microorganisms present in all the media used after 0, 60 and 180 days of the start of the fermentation process

Table 4.6 - Average cell counts (as well as the standard deviation) for moulds, yeasts and bacteria in all media used after 0, 60 and 180 days of the start of the fermentation process for grass pea miso inoculated with *Candida versatilis*.

	Moulds		**Yeasts**		**Bacteria**	
	Mean (CFU/mL)	**Standard deviation (CFU/mL)**	**Mean (CFU/mL)**	**Standard deviation (CFU/mL)**	**Mean (CFU/mL)**	**Standard deviation (CFU/mL)**
0 days	$1.54x10^5$	$5.92x10^4$	$2.50x10^3$	$2.12x10^3$	$1.56x10^5$	$1.25x10^5$
60 days	$2.05x10^2$	$1.55x10^2$	$5.10x10^4$	$3.64x10^4$	$7.53x10^4$	$2.23x10^4$
180 days	$<1.00\ x10^3$	0	$9.57x10^4$	$1.39x10^4$	$<1.00\ x10^3$	0

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Table 4.6 shows that the mould population cell counts are high in the early stages of maturation but starts to decrease in the 2nd month of fermentation. Even though there are not many studies in the miso's microbiota area, the same variation happens on soy sauce (Xu, et al., 2016) and *doenjang* (Kum, et al., 2015) which are 2 fermented soybean products.

The yeast population increases throughout the 6 months of microbiota development. The cell counts start at 10^3 CFU/mL and increase during the fermentation process to 10^4 CFU/mL after 2 months. This same kind of increase was reported in a previous study regarding the analysis of bacterial flora in miso fermentation (Onda, Yanagida, Tsuji, Shinohara, & Yokotsuka, 2003).

The bacterial population decreases throughout the 6 months of microbiota development. The cell counts start high (10^5 CFU/mL) and decrease during the fermentation process to 10^4 CFU/mL after 2 months. This same kind of decrease was reported in a previous study regarding the analysis of bacterial flora in miso fermentation (Onda, Yanagida, Tsuji, Shinohara, & Yokotsuka, 2003).

In the first days of the fermentation process, there were some yellow and transparent bacteria which disappeared after the 1st month of fermentation.

4.4. Identification of the microorganisms by molecular methods

4.4.1. Samples from the fermentation of miso

4.4.1.1. Yeast isolates

After all the plates are fully grown, some isolated colonies from each media were selected to be sequenced and analyzed, based on their different macromorphology.

Table 4.7 shows all the samples selected from the 3rd,4th and 5th month of fermentation of miso batch of fully-grown plates and their macromorphological characteristics:

Table 4.7 - Selected samples of the yeasts isolates from the fermentation of miso

Sample number	Type of miso	Starter(s)	Media
C1_3	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
C2_3	Grass pea	*C. versatilis* and *Z. rouxii*	MRS
C3_3	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
C4_3	Grass pea	*Candida versatilis*	MRS
C5_3 ①	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
C5_3 ②	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
C6_3	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
C7_3	Grass pea	*Candida versatilis*	YPD+10% NaCl
C8_3	Grass pea	*C. versatilis* and *Z. rouxii*	YPD+10% NaCl
C9_3	Grass pea	*C. versatilis* and *Z. rouxii*	MRS+10% NaCl
C10_3	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
C11_3	Grass pea	*C. versatilis* and *Z. rouxii*	YPD+10% NaCl
C12_3	Soybean	*Z. rouxii*	YPD+10% NaCl
C1_4	Grass pea	*Z. rouxii*	MRS+10% NaCl
C2_4	Grass pea	*Z. rouxii*	YPD
C3_4 ①	Grass pea	*Z. rouxii*	MRS
C3_4 ②	Grass pea	*Z. rouxii*	MRS
C4_4	Grass pea	*Z. rouxii*	YPD
C5_4	Grass pea	*Z. rouxii*	YPD+10% NaCl
C6_4	Soybean	*Z. rouxii*	YPD
C7_4	Grass pea	*C. versatilis* and *Z. rouxii*	MRS+10% NaCl
C8_4	Grass pea	*C. versatilis* and *Z. rouxii*	YPD+10% NaCl
C9_4	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
C10_4 ①	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
C10_4 ②	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
C11_4 ①	Soybean	*C. versatilis* and *Z. rouxii*	YPD
C11_4 ②	Soybean	*C. versatilis* and *Z. rouxii*	YPD

C12_4	Grass pea	*Candida versatilis*	YPD
C13_4	Grass pea	*Candida versatilis*	MRS+10% NaCl
C14_4	Soybean	*Candida versatilis*	YPD
C1_5 ①	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
C1_5 ②	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
C2_5	Grass pea	*Z. rouxii*	YPD
C3_5 ①	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
C3_5 ②	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
C4_5	Soybean	*C. versatilis* and *Z. rouxii*	YPD
C5_5	Grass pea	*C. versatilis* and *Z. rouxii*	YPD+10% NaCl
C6_5	Grass pea	*Z. rouxii*	YPD+10% NaCl
C7_5	Grass pea	*C. versatilis* and *Z. rouxii*	MRS
C8_5	Grass pea	*C. versatilis* and *Z. rouxii*	MRS+10% NaCl
C9_5	Grass pea	*Candida versatilis*	MRS+10% NaCl
C10_5	Grass pea	*Z. rouxii*	MRS+10% NaCl

The macromorphology of these previous samples showed white raised colonies.

The DNA from each colony was isolated according to described in section 3.6.4.1.

4.4.1.2. Bacterial isolates

After all the plates are fully grown, some isolated colonies from each media were selected to be sequenced and analyzed, based on their different macromorphology.

Table 4.8 shows all the samples selected from the 3rd,4th and 5th month of fermentation of miso batch of fully-grown plates and their macromorphological characteristics:

Table 4.8 - Selected samples of the bacterial isolates from the fermentation of miso

Sample number	Type of miso	Starter(s)	Media
BC3_3	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
BC5_3 ①	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
BC4_4	Grass pea	*Z. rouxii*	YPD
BC6_4	Soybean	*Z. rouxii*	YPD
BC10_4 ①	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
BC11_4 ②	Soybean	*C. versatilis* and *Z. rouxii*	YPD
BC12_4	Grass pea	*Candida versatilis*	YPD
BC14_4	Soybean	*Candida versatilis*	YPD
BC1_5 ②	Grass pea	*C. versatilis* and *Z. rouxii*	YPD

BC3_5 ②	Grass pea	*C. versatilis* and *Z. rouxii*	YPD
BC4_5	Soybean	*C. versatilis* and *Z. rouxii*	YPD

The macromorphology of these previous samples showed a rod-shaped bacterium.

The DNA from each colony was isolated according to described in section 3.6.4.1.

4.4.2. Electrophoresis

4.4.2.1. Yeast isolates

To confirm the presence and quantify the DNA from all the samples, an agarose gel electrophoresis (0,8% agarose) was run (Figure 4.6).

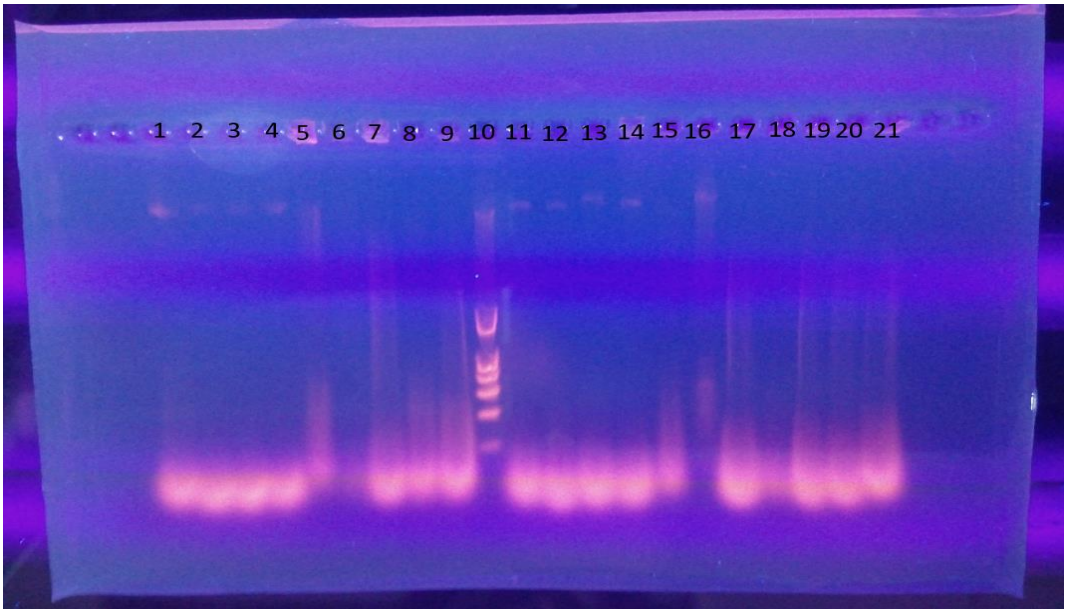

Figure 4.6 - Agarose gel electrophoresis (0,8% agarose) of DNA quantification after DNA extraction of the samples from the 4th month of fermentation of miso. Lanes 1-4 (C1_4), lane 5 (C2_4), lane 6 (C3_4①), lane 7 (C4_4), lane 8 (C5_4), lane 9 (C6_4), lanes 11-14 (C7_4), lane 15 (C8_4), lane 16 (C9_4), lane 17 (C10_4①), lanes 18-19 (C11_4), lane 20 (C12_4) and lane 21 (C14_4) are miso sample. Lane 10 is λHindIII size marker.

As shown in figure 4.6, it is possible to see the presence of both DNA and RNA from the miso samples. It is also possible to see the degradation of the DNA of some samples (lanes 5,7,8,9,15,16,17,19,20,21) through the smearing shown between the DNA and RNA. The presence of DNA is shown in lanes 1,2,3,4,5,11,12,13,14 and 16.

Once we have the confirmation shown previously, PCR reactions were performed with specific primers (see section 3.6.4.2.) in order to amplify the ITS4-ITS5 DNA region (regarding yeasts). After the PCR reaction, an agarose gel electrophoresis (0.8% agarose) was run to confirm that the desired fragments of DNA were amplified (Figure 4.7).

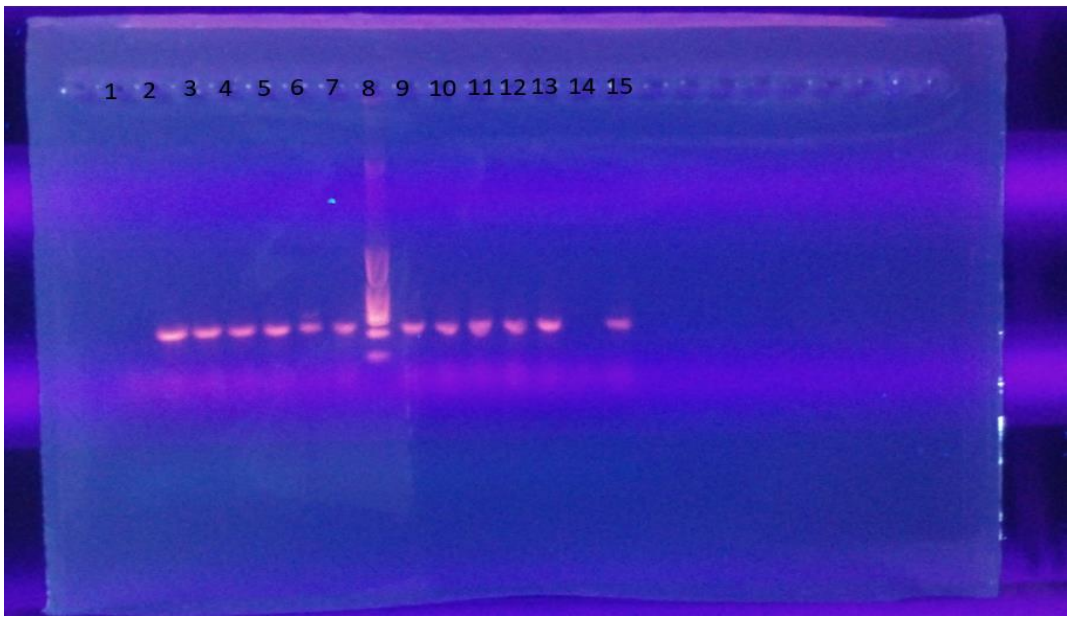

Figure 4.7 - Agarose gel electrophoresis (0,8% agarose) of PCR amplified products of the samples from the 4th month of fermentation of miso. Lane 1 is a negative control sample. Lanes 2-5 (C1_4), lane 6 (C2_4), lane 7 (C5_4), lanes 9-12 (C7_4), lane 13 (C8_4), lane 14 (C9_4) and lane 15 (C11_4①) are miso samples. Lane 8 is NZYDNA ladder I size marker.

As shown in figure 4.7, it's visible the amplification of our DNA samples by the polymerase chain reaction. The DNA band is visible in all samples except sample number 1 (negative control) and sample 14 where it wasn't amplified.

With the 5-month yeast samples, after the PCR, there wasn't any type of amplification, so the annealing temperature was reduced (from 49ºC to 48ºC). This was also not enough because not many samples exhibited amplification. After, the master mix was changed by adding 1 µL of the DNA sample and by reducing the amount of MiliQ water used by the same amount. This was not also enough and therefore the annealing temperature was changed again (from 48ºC to 47ºC).

Before our samples are sequenced, they were purified by the wizard columns method (described in section 3.6.4.3) and let to concentrate by evaporation of water for a day, using a vacuum desiccator. An agarose gel electrophoresis (1,5% agarose) was run to quantify the obtained DNA (Figure 4.8).

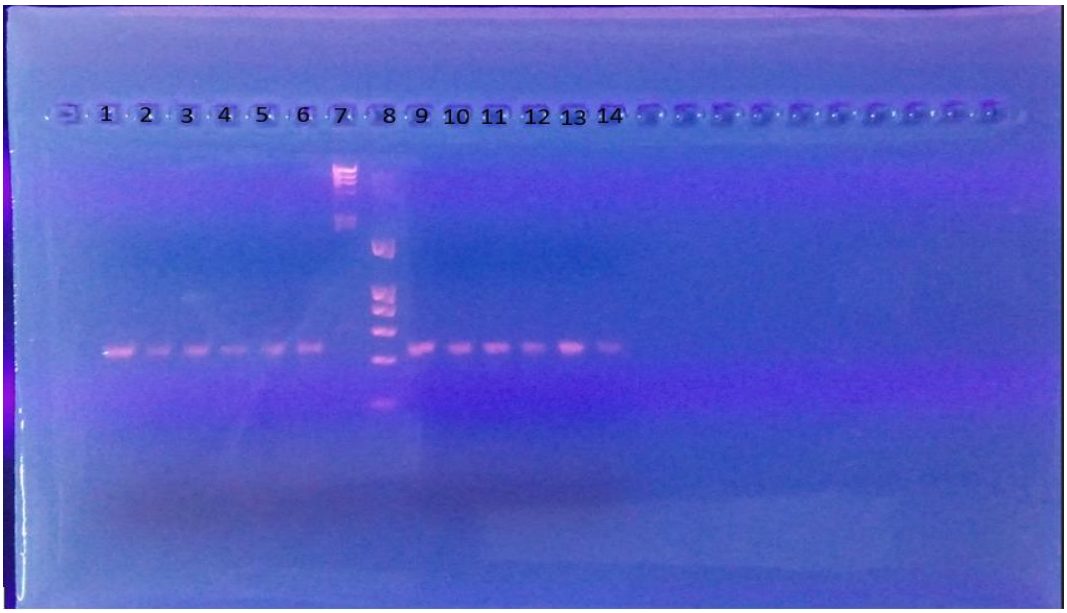

Figure 4.8 - Agarose gel electrophoresis (1,5% agarose) of PCR amplified products after purification of the samples from the 4th month of fermentation of miso. Lanes 1-4 (C1_4), lane 5 (C2_5), lane 6 (C5_4), lanes 9-12 (C7_4), lane 13 (C8_4) and lane 14 (C11_4) are miso's samples. Lane 7 and 8 are λHindIII size marker and NZYDNA ladder I size marker, respectively.

As shown in figure 4.8, it's visible the bands that were present in the PCR amplification electrophoresis (figure 4.7) are still there and so these samples are ready for sequencing.

The samples having enough DNA concentration were then sent to be sequenced. In the cases in which no DNA amplification was observed, we repeated the DNA extraction and PCR reaction lowering the annealing temperature by one degree.

In some cases, the amount of amplified DNA was not enough to be sequenced so a concentration needed to be done. Most of the electrophoresis after the concentration showed no sign of purified DNA. The purification method was changed slightly to make sure all the DNA got attached to the minicolumn and then removed properly from it. It was necessary to use the centrifuge twice after the DNA was attached to the column and purified instead of the one time shown on the protocol given by the kit.

4.4.2.2. Bacterial isolates

To confirm the presence and quantify the DNA from all the samples, an agarose gel electrophoresis (0,8% agarose) was run (Figure 4.9).

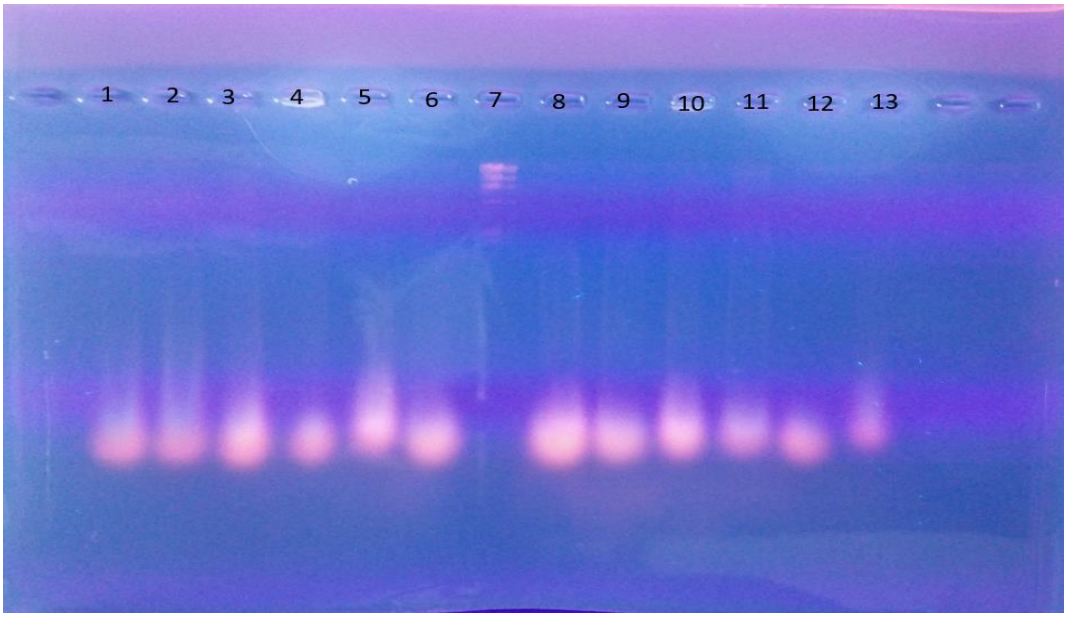

Figure 4.9 – Agarose gel electrophoresis (0,8% agarose) of DNA quantification after DNA extraction of the bacteria samples selected from the 3rd,4th and 5th month of fermentation of miso. Lane 1 (BC3_3), lane 2 and 3 (BC5_3), lane 4 (BC4_4), lane 5 (BC6_4), lane 6 (BC10_4①), lane 8 (BC11_4), lane 9 (BC12_4), lane 10 (BC14_4), lane 11 (BC1_5), lane 12 (BC3_5), lane 13 (BC4_5) are miso samples. Lane 7 is λHindIII size marker.

As shown in figure 4.9, it is possible to see the presence of both DNA and RNA from the miso samples. It is also possible to see the degradation of the DNA of some samples (lanes 1,2,3,5,8,9,10,11 and 12) through the smearing shown between the DNA and RNA. The presence of DNA is more difficult to see but there are some traces in lanes 1,2,3 and 10.

Once we have the confirmation shown previously, PCR reactions were performed with specific primers (see section 3.6.4.2.) in order to amplify and D1-D2 DNA region (in the case of DNA extracted from bacterial colonies). The amount of bacteria DNA used to do the PCR master mix was 3 µL instead of 1 µL. After the PCR reaction, an agarose gel electrophoresis (0,8% agarose) was run to confirm that the desired fragments of DNA were amplified (Figure 4.10):

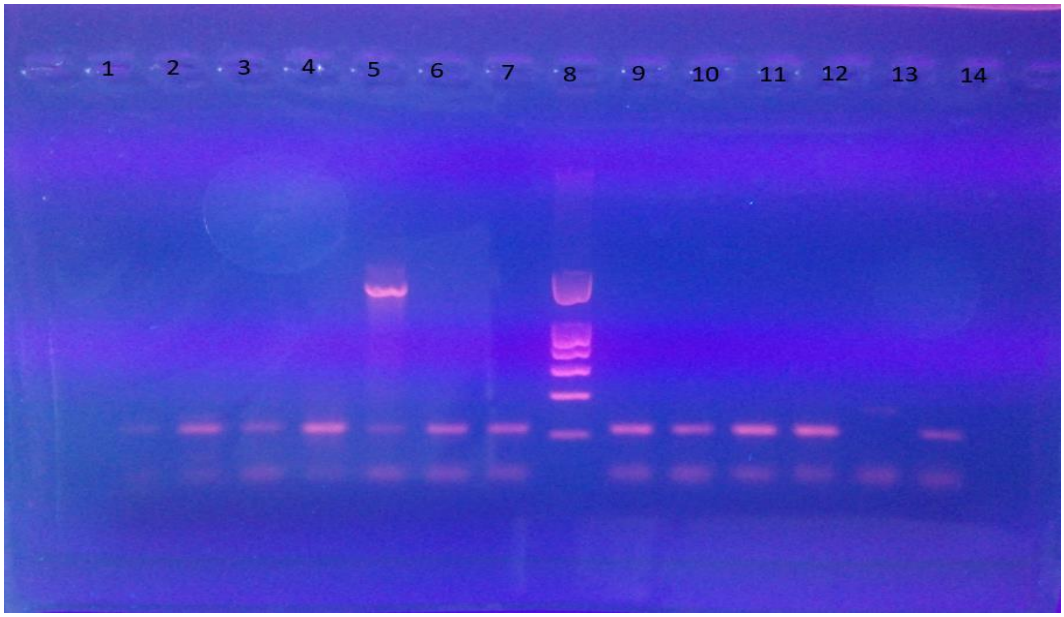

Figure 4.10 - Agarose gel electrophoresis (0,8% agarose) of PCR amplified products of the bacteria samples selected from the 3rd,4th and 5th month of fermentation of miso. Lane 1 is a negative control sample. Lanes 2 (BC3_3), lane 3 and 4 (BC5_3), lane 5 (BC4_4), lane 6 (BC6_4), lane 7 (BC10_4①), lane 9 (BC11_4), lane 10 (BC12_4), lane 12 (BC14_4), lane 13 (BC1_5), lane 13 (BC3_5), lane 14 (BC4_5) are miso samples -7 and 9-14 are miso's samples. Lane 8 is NZYDNA ladder I size marker.

As shown in figure 4.10, it's visible the amplification of our DNA samples by the polymerase chain reaction. The DNA band (≈ 450 bp) is visible in all samples except sample number 1 (negative control) and sample 13 where it wasn't amplified. By comparing the other amplifications with the sample 5, it shows a contamination with 1800 bp.

In this part of the work, not all the samples were amplified so it was necessary to do several reamplifications. Firstly, it was used 1 µL of each of the DNA samples and it didn't work. This amount was then changed to 2 µL and there was enough DNA after amplification to be purified.

Before our samples are sequenced, they were purified by the wizard columns method (described in section 3.6.4.3). An agarose gel electrophoresis (1,5% agarose) was run to quantify the obtained DNA (Figure 4.11).

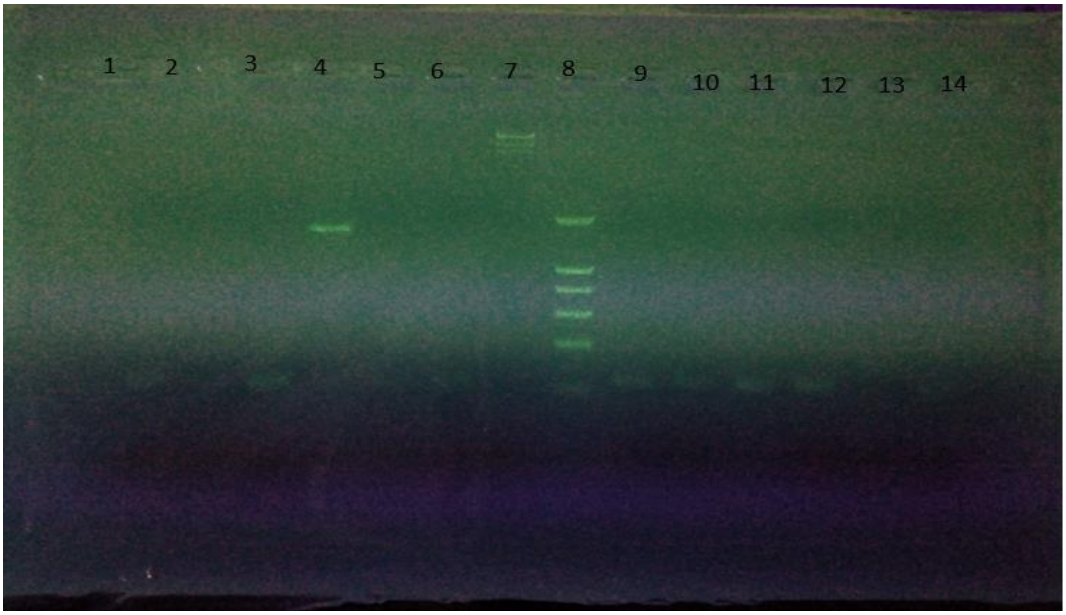

Figure 4.11 - Agarose gel electrophoresis (1,5% agarose) of PCR amplified products after purification of the bacteria samples selected from the 3rd,4th and 5th month of fermentation of miso. Lanes 1 (BC3_3), lane 2 and 3 (BC5_3), lane 4 (BC4_4), lane 5 (BC6_4), lane 6 (BC10_4①), lane 9 (BC11_4), lane 10 (BC12_4), lane 12 (BC14_4), lane 13 (BC1_5), lane 13 (BC3_5), lane 14 (BC4_5) are miso samples. Lanes 7 and 8 are λHindIII size marker and NZYDNA ladder I size marker, respectively.

As shown in figure 4.11, it's visible the bands that were present in the PCR amplification electrophoresis (figure 4.10) are still there and so these samples are ready for sequencing.

The samples having enough DNA concentration were then sent to be sequenced. In the cases in which no DNA amplification was observed, we repeated the DNA extraction and PCR reaction lowering the annealing temperature by one degree and sometimes increasing the amount of DNA samples on the PCR master mix.

4.5. Sequencing analysis using the BLAST search engine

4.5.1. Yeast isolates

As it was visible in the electrophoresis before, not all samples could have been used for sequencing. In the table below, it's shown all the samples that were sequenced, and then analyzed using the BLAST search engine:

Table 4.9 - Sequencing results from all the yeast samples from 3rd, 4th and 5th month of the fermentation of miso.

Sample number	Type of miso	Starter(s)	Sequenced microorganism	Identity
C1_3	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	98%
C7_3	Grass pea	*Candida versatilis*	*Candida versatilis*	99%
C8_3	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C9_3	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C11_3 ①	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	100%
C11_3②	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	100%
C2_4	Grass pea	*Z. rouxii*	*Candida versatilis*	99%
C5_4	Grass pea	*Z. rouxii*	*Candida versatilis*	99%
C7_4 ①	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C7_4 ②	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C7_4 ③	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C7_4 ④	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C8_4	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C11_4 ①	Soybean	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C11_4 ②	Soybean	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C12_4	Grass pea	*Candida versatilis*	*Candida versatilis*	99%
C13_4	Grass pea	*Candida versatilis*	*Candida versatilis*	99%
C14_4	Soybean	*Candida versatilis*	*Candida versatilis*	99%
C5_5	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C6_5	Grass pea	*Z. rouxii*	*Candida versatilis*	99%
C7_5 ②	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C7_5 ③	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C7_5 ④	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C8_5 ②	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C8_5 ③	Grass pea	*C. versatilis* and *Z. rouxii*	*Candida versatilis*	99%
C10_5	Grass pea	*Z. rouxii*	*Candida versatilis*	99%

After analyzing all the yeast's sequences with the help of the BLAST search engine, it was possible to see that the yeasts that were supposed to be *Zygosaccharomyces rouxii* were instead *Candida versatilis*. This might have been caused by mixing samples while extracting the DNA, during the PCR procedure or the purification. Another possibility is that the sample of *Zygosaccharomyces rouxii* was not correct in the ISA collection and was instead *Candida versatilis*.

4.5.2. Bacterial isolates

As it was visible in the electrophoresis before, not all samples could have been used for sequencing. In the table below, it's shown all the samples that were sequenced, and then analyzed using the BLAST search engine:

Table 4.10 – Sequencing results from the bacteria samples from the 3rd,4th and 5th month of the fermentation of miso.

Sample number	Type of miso	Starter(s)	Sequenced microorganism	Identity
C3_3	Grass pea	*C. versatilis* and *Z. rouxii*	*Bacillus sp.*	98%
C5_3 ①	Grass pea	*C. versatilis* and *Z. rouxii*	*Bacillus sp.*	99%
C5_3 ②	Grass pea	*C. versatilis* and *Z. rouxii*	*Bacillus sp.*	99%
C4_4	Grass pea	*Z. rouxii*	*Bacillus sp.*	99%
C6_4	Soybean	*Z. rouxii*	*Bacillus subtilis*	100%
C10_4 ①	Grass pea	*C. versatilis* and *Z. rouxii*	*Bacillus sp.*	99%
C11_4 ①	Soybean	*C. versatilis* and *Z. rouxii*	*Bacillus sp.*	100%
C12_4	Grass pea	*Candida versatilis*	*Bacillus cereus*	99%
C14_4	Soybean	*Candida versatilis*	*Bacillus sp.*	99%
C1_5 ②	Grass pea	*C. versatilis* and *Z. rouxii*	*Bacillus sp.*	99%
C3_5 ②	Grass pea	*C. versatilis* and *Z. rouxii*	*Bacillus sp.*	99%
C4_5	Soybean	*C. versatilis* and *Z. rouxii*	*Bacillus cereus*	99%

4.6. Challenge tests

4.6.1. Grass pea miso sample inoculated with *Candida versatilis* and *Zygosaccharomyces rouxii*

With the help of their respective calibration curves it was possible to make a solution containing five pathogenic microorganisms: *Bacillus cereus*, *Escherichia coli*, *Listeria innocua*, *Salmonella enterica Typhimurium* and *Staphylococcus aureus*. This solution was then added to our grass pea miso samples (with no fermentation and 7 months of fermentation) and left to incubate at three different temperatures: 37ºC, room temperature and 4ºC. Samples were then collected with different time intervals (0, 2, 4, 7, 14, 30 and 60 days) and inoculated in specific plated media.

4.6.1.1. *Bacillus cereus*

For the counting of *Bacillus cereus*, the media was *Bacillus cereus* agar (base acc. To Mossel) *Biokar Diagnostics*, and the samples were collected after 0, 2, 4, 7, 14, 30 and 60 days of inoculation. For both control grass pea miso and for grass pea miso with 7 months of fermentation the samples were collected during 60 days.

The results shown below showcase the evolution of *Bacillus cereus* lack of growth within the three different temperatures for 60 days.

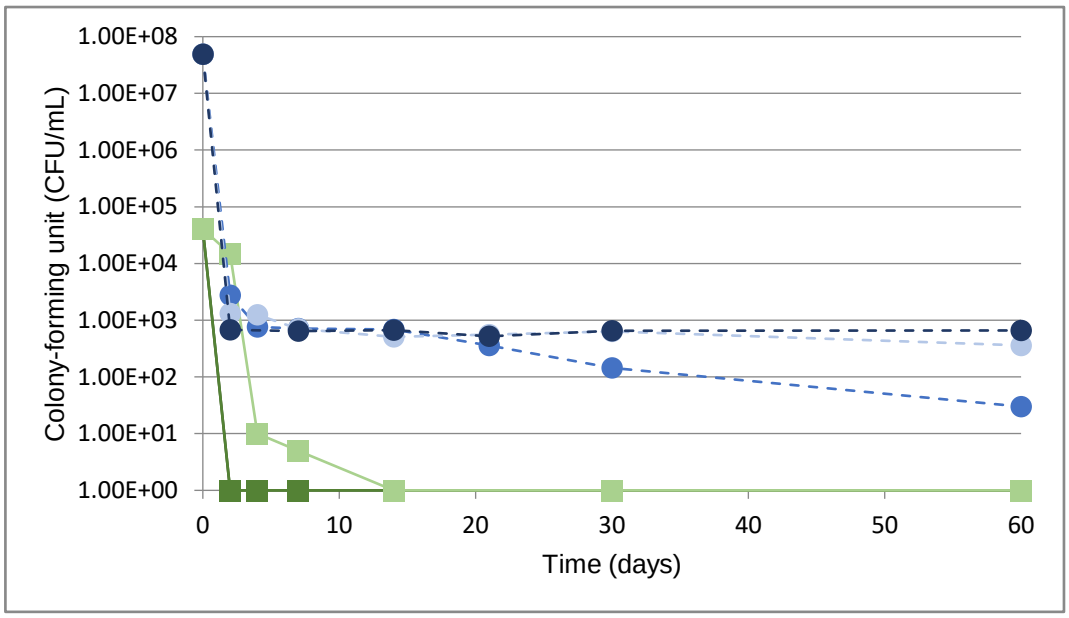

Figure 4.12 - Grass pea miso (*Candida versatilis* and *Zygosaccharomyces rouxii*) collected samples for both control miso (solid line) and with 7 months of fermentation (dash line) regarding the growth of *Bacillus cereus*. The solid lines represent the evolution of the control grass pea miso during 30 days in three different temperatures: in the oven (37ºC), at room temperature (≈25ºC) and in the fridge (4ºC). The dash lines represent the evolution of the grass pea miso with 7 months of fermentation during 60 days in three different temperatures: in the oven (37ºC), at room temperature (≈25ºC) and in the fridge (4ºC). Actual numbers of cells/mL are presented on a logarithmic scale (Y axis).

The lack of growth of *Bacillus cereus* is visible by looking at the figure 4.12. In the control grass pea miso sample, it's possible to see that the population of *Bacillus cereus* got eliminated after 2 days while stored at the temperatures of 37ºC and 25ºC, but it needed 14 days to be eliminated while stored in the fridge (4ºC). The same didn't happen with the grass pea miso sample with 7 months of fermentation where it's possible to see that the population decreases but it never gets eliminated. This is due to the fact that since *Bacillus cereus* produces endospores, when it's inoculated in a favorable medium these start to germinate. The decrease in the *Bacillus cereus* population was expected since it has been reported that at a 10% NaCl content this bacterium doesn't grow (Raevuori & Genigeorgis, 1975).

4.6.1.2. *Escherichia coli*

For the counting of *Escherichia coli*, the media was Compass ECC agar, *Biokar Diagnostics*, and the samples were collected after 0, 2, 4, 7, 14, 30 and 60 days of inoculation. For both control grass pea miso and for grass pea miso with 7 months of fermentation the samples were collected during 60 days.

The results shown below showcase the evolution of *Escherichia coli* lack of growth within the three different temperatures for 60 days.

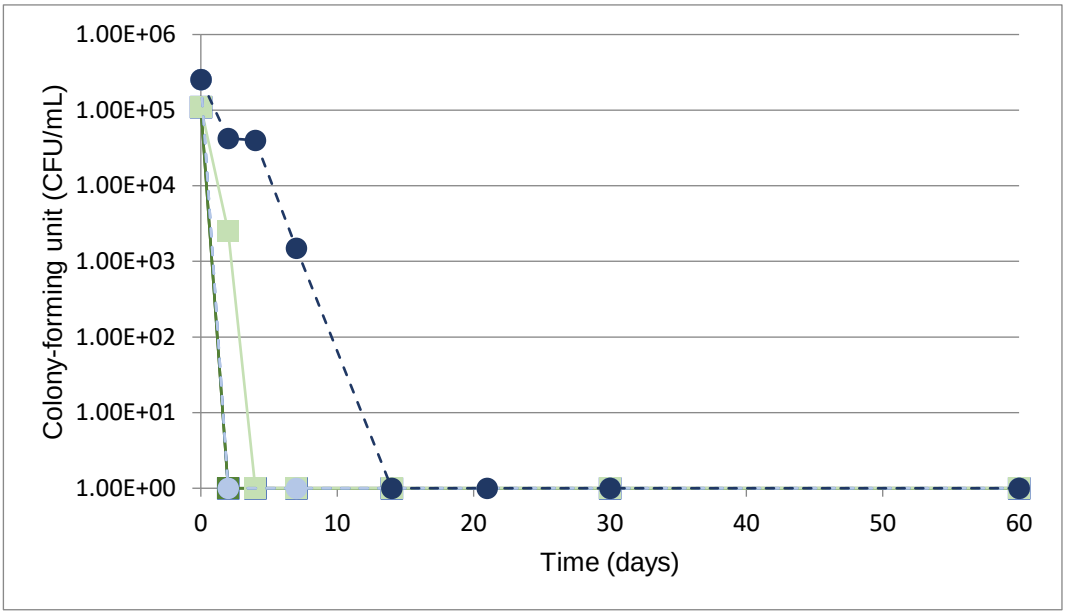

Figure 4.13 - Grass pea miso (*Candida versatilis* and *Zygosaccharomyces rouxii*) collected samples for both control miso (solid line) and with 7 months of fermentation (dash line) regarding the growth of *Escherichia coli*. The solid lines represent the evolution of the control grass pea miso during 30 days in three different temperatures: in the oven (37ºC), at room temperature (≈25ºC) and in the fridge (4ºC). The dash lines represent the evolution of the grass pea miso with 7 months of fermentation during 60 days in three different temperatures: in the oven (37ºC), at room temperature (≈25ºC) and in the fridge (4ºC). Actual numbers of cells/mL are presented on a logarithmic scale (Y axis).

The lack of growth *Escherichia coli* is visible by looking at the figure 4.13. In the control grass pea miso sample, it's possible to see that the *Escherichia coli* population got completely eliminated after 2 days while stored at the temperatures of 37ºC and 25ºC, but it needed 4 days to be eliminated while stored in the fridge (4ºC). In the grass pea miso sample with 7 months of fermentation the *Escherichia coli* population got eliminated after 2 days in the samples stored at the temperatures of 37ºC and 25ºC but it needed 14 days to be eliminated while stored in the fridge (4ºC). The reduction of the E*scherichia coli* population was expected since at a content of 1,0% (w/w) of NaCl, it has been reported that the population started decreasing (Abdulkarim, Fatimah, & Anderson, 2009).

4.6.1.3. *Listeria innocua*

For the counting of *Listeria innocua*, the media was PALCAM agar (base), *Biokar Diagnostics*, and the samples were collected after 0, 2, 4, 7, 14, 30 and 60 days of inoculation. For both control grass pea miso and for grass pea miso with 7 months of fermentation the samples were collected during 60 days.

The results shown below showcase the evolution of *Listeria innocua* lack of growth within the three different temperatures for 60 days.

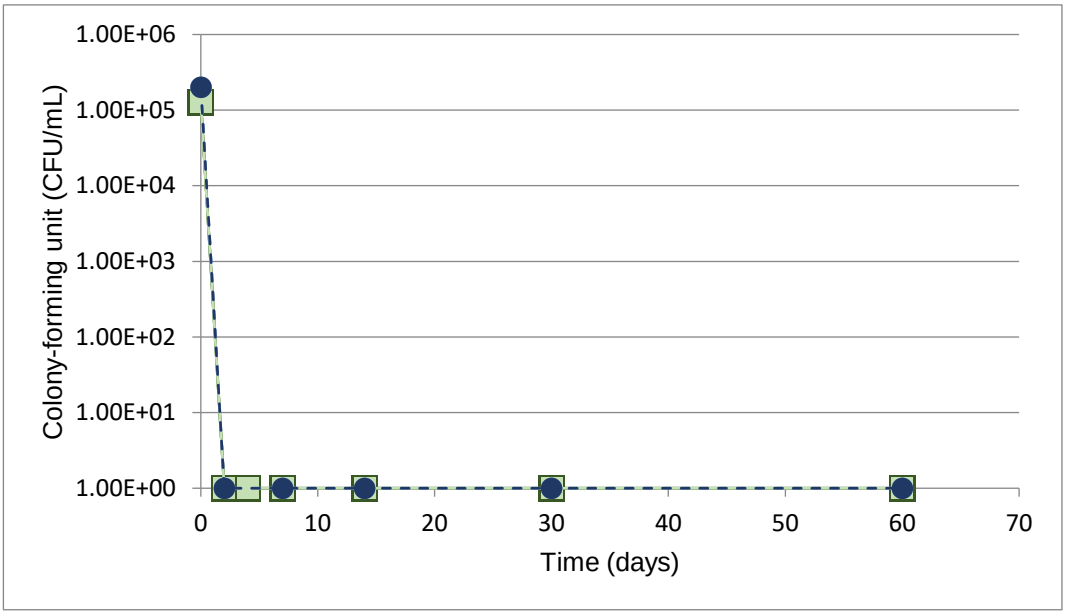

Figure 4.14 - Grass pea miso (*Candida versatilis* and *Zygosaccharomyces rouxii*) collected samples for both control miso (solid line) and with 7 months of fermentation (dash line) regarding the growth of *Listeria innocua*. The solid lines represent the evolution of the control grass pea miso during 30 days in three different temperatures: in the oven (37ºC), at room temperature (≈25ºC) and in the fridge (4ºC). The dash lines represent the evolution of the grass pea miso with 7 months of fermentation during 60 days in three different temperatures: in the oven (37ºC), at room temperature (≈25ºC) and in the fridge (4ºC). Actual numbers of cells/mL are presented on a logarithmic scale (Y axis).

The lack of growth *Listeria innocua* is visible by looking at the figure 4.14. In both the grass pea miso samples it's possible to see the immediate elimination after 2 days of all traces of *Listeria innocua* in all three temperatures. Though this bacterium shows high survivability under high concentrations of salt, its growth is inhibited by this external stress (Liu, Lawrence, Ainsworth, & Austin, 2005) and once it's removed the growth goes back to normal.

4.6.1.4. *Salmonella enterica Typhimurium*

For the counting of *Salmonella enterica Typhimurium*, the media was XLD agar (ISO 6579), *Biokar Diagnostics*, and the samples were collected after 0, 2, 4, 7, 14, 30 and 60 days of inoculation. For both control grass pea miso and for grass pea miso with 7 months of fermentation the samples were collected during 60 days.

The results shown below showcase the evolution of *Salmonella enterica Typhimurium* lack of growth within the three different temperatures for 60 days.

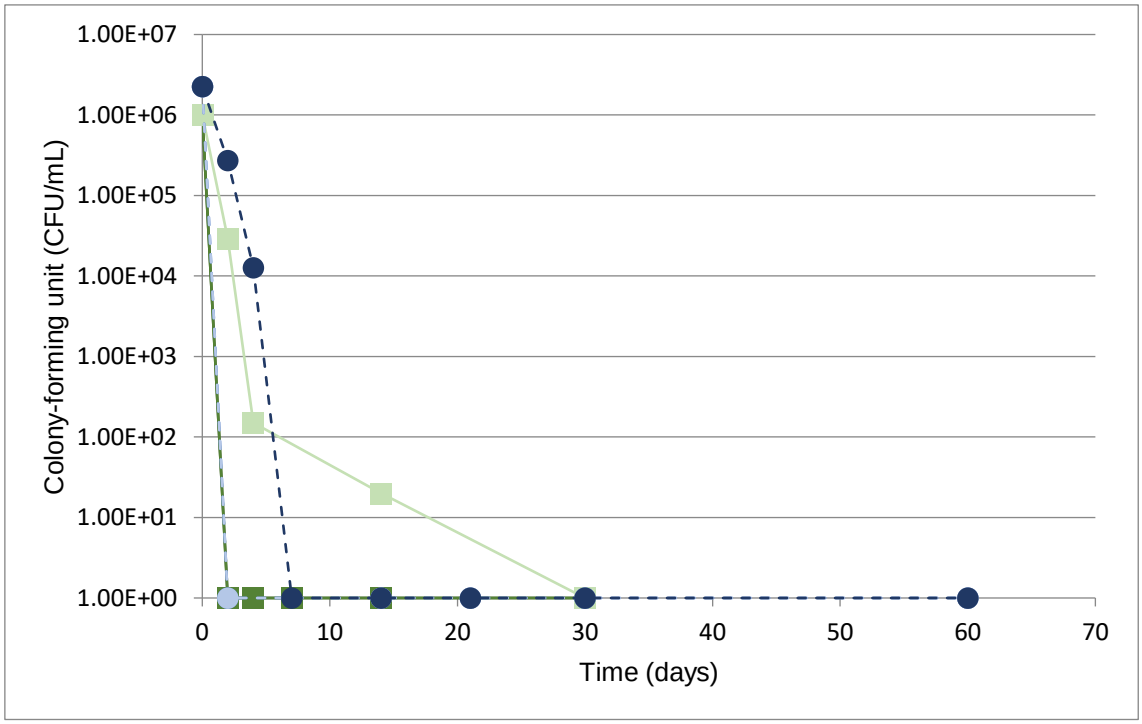

Figure 4.15 - Grass pea miso (*Candida versatilis* and *Zygosaccharomyces rouxii*) collected samples for both control miso (solid line) and with 7 months of fermentation (dash line) regarding the growth of *Salmonella enterica Typhimurium*. The solid lines represent the evolution of the control grass pea miso during 30 days in three different temperatures: in the oven (37ºC), at room temperature (≈25ºC) and in the fridge (4ºC). The dash lines represent the evolution of the grass pea miso with 7 months of fermentation during 60 days in three different temperatures: in the oven (37ºC), at room temperature (≈25ºC) and in the fridge (4ºC). Actual numbers of cells/mL are presented on a logarithmic scale (Y axis).

The lack of growth *Salmonella enterica Typhimurium* is visible by looking at the figure 4.15. In the control grass pea miso sample, it's possible to see that the *Salmonella enterica Typhimurium* population got eliminated after 2 days while it was stored at the temperatures of 37ºC and 25ºC, but it needed a bit more time (30 days) to be eliminated while it was stored at 4ºC. In the grass pea miso sample with 7 months of fermentation the *Salmonella enterica Typhimurium* population got eliminated after 2 days in the samples stored at the temperatures of 37ºC and 25ºC but it needed 7 days to be eliminated while stored in the fridge (4ºC). The decrease in the *Salmonella enterica Typhimurium* population is related to the salt content of miso. In the presence of high concentrations of salt, the growth of this bacteria is decreased (Matches & Liston, 1972).

4.6.1.5. *Staphylococcus aureus*

For counting of *Staphylococcus aureus*, the media was Gelose Baird-Parker (base), *Biokar Diagnostics*, and the samples were collected after 0, 2, 4, 7, 14, 30 and 60 days of inoculation. For both control grass pea miso and for grass pea miso with 7 months of fermentation the samples were collected during 60 days.

The results shown below showcase the evolution of *Staphylococcus aureus* lack of growth within the three different temperatures for 60 days.

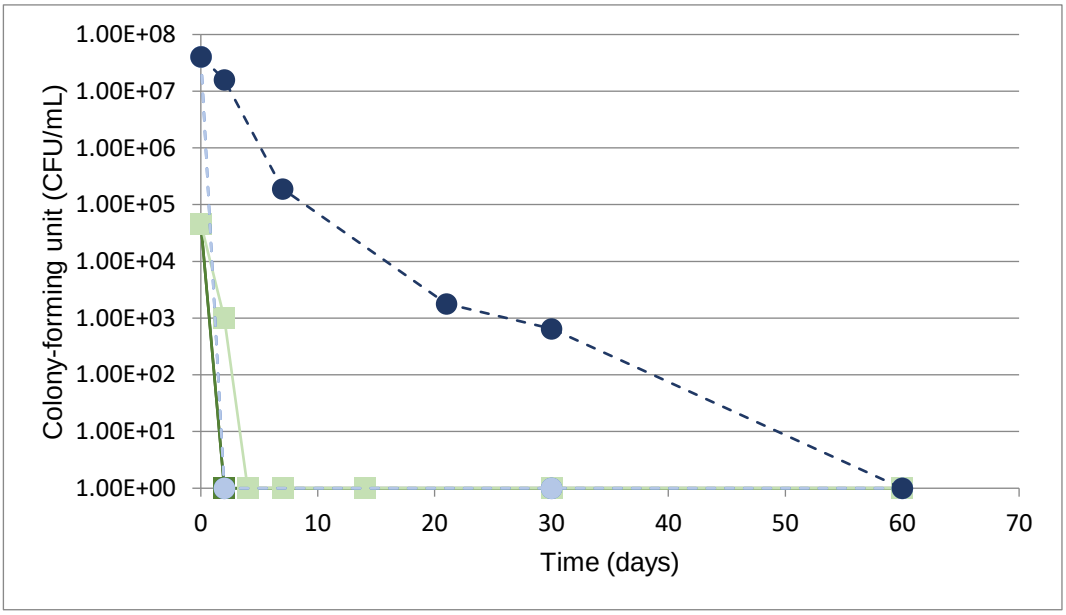

Figure 4.16 - Grass pea miso (*Candida versatilis* and *Zygosaccharomyces rouxii*) collected samples for both control miso (solid line) and with 7 months of fermentation (dash line) regarding the growth of *Staphylococcus aureus*. The solid lines represent the evolution of the control grass pea miso during 30 days in three different temperatures: in the oven (37ºC), at room temperature (≈25ºC) and in the fridge (4ºC). The dash lines represent the evolution of the grass pea miso with 7 months of fermentation during 60 days in three different temperatures: in the oven (37ºC), at room temperature (≈25ºC) and in the fridge (4ºC). Actual numbers of cells/mL are presented on a logarithmic scale (Y axis).

The lack of growth *Staphylococcus aureus* is visible by looking at the figure 4.16. In the control grass pea miso sample, it's possible to see that the *Staphylococcus aureus* population got eliminated after 2 days while it was stored at the temperatures of 37ºC and 25ºC, but it took 4 days to be eliminated while it was stored at 4ºC.

In the grass pea miso sample with 7 months of fermentation the *Staphylococcus aureus* population got eliminated after 2 days in the samples stored at the temperatures of 37ºC and 25ºC, but it needed 60 days to be eliminated while stored in the fridge (4ºC). For *Staphylococcus aureus* the growth is less effective when higher temperatures are combined with high salt contents (Smolka, Nelson, & Kelley, 1974) which makes the results at 4ºC more expected.

5. Conclusions and future perspectives

Regarding miso's microbiota we can conclude that *Candida versatilis* maintains more viability in the grass pea miso. The existence of lactic acid bacteria is very noticeable in both types misos which means the fermentation process is going as expected. *Aspergillus oryzae* is only detected in the beginning of the fermentation which is also normal because their role in the fermentation process is done. Only after the DNA analysis of some samples, it was possible to realize that the results were only about *Candida versatilis* and not with *Zygosaccharomyces rouxii*.

After analyzing the color evolution results, it's possible to conclude that the color of miso evolves more slowly when a starter culture is used (being soybean or grass pea). One of the main reasons for this slow evolution it's due the fact that the starter culture is not yet optimized. If the study had prolonged for more time than 6 months, it would eventually reach the same color scheme as the traditional miso.

Regarding the challenge test studies, it was possible to conclude that the fact that the miso is fermented doesn't contribute to the elimination of the pathogenic microorganisms introduced. This study was made with a grass pea miso with no fermentation and 7 months of fermentation and both showed the capacity to eliminate all pathogens inoculated.

The best temperature to store grass pea miso is 25ºC (room temperature) or 37ºC because it doesn't allow the growth of pathogenic microorganisms and their elimination is very effective in case of contamination.

In the future, there needs to be a lot of study surrounding this topic because it's a vast topic that needs some "exploration". It is important to develop a starter culture that tries to replicate the traditional miso complex microbiota conditions but also its colors. Even though we have not yet developed the perfect starter culture, the miso done with this starter culture has been evaluated by Sense Test (Vila Nova de Gaia, Portugal) and characterized as having a clear appearance, blond color, soft and salty taste and a velvety texture (personal communication). This recipe of miso has been presented in some festivals, especially during the grass pea festival in Alvaiázere (Leiria, Portugal) and it has been receiving quite nice feedback.

It would also be important to do a detailed biochemical and nutritional characterization of the grass pea miso in order to know more about what happens during fermentation (e.g. how lipids work). Though it's plausible to think that the NaCl content might be the factor behind the

elimination of the pathogenic microorganisms, it would also be important to either confirm that or search for other factors responsible for this elimination.

6

6. Bibliography

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7. Appendix

7

Appendix 1 – Graphic values of grass pea miso sample (*Candida versatilis* and *Zygosaccharomyces rouxii*) with no fermentation for each pathogenic microorganism.

Bacillus cereus

Table 7.1 - Graphic values for *Bacillus cereus* in the grass pea miso sample (*Candida versatilis* and *Zygosaccharomyces rouxii*) with no fermentation stored at a temperature of 37ºC (A), room temperature (B) and at a temperature of 4ºC (C).

(A)

Time (days)	CFU/mL
0	4,03E+04
2	0,00E+00
4	0,00E+00
7	0,00E+00
14	0,00E+00
30	0,00E+00

(B)

Time (days)	CFU/mL
0	4,03E+04
2	0,00E+00
4	0,00E+00
7	0,00E+00
14	5,00E+00
30	0,00E+00

(C)

Time (days)	CFU/mL
0	4,03E+04
2	1,50E+03
4	0,00E+00
7	5,00E+00
14	0,00E+00
30	0,00E+00

Escherichia coli

Table 7.2 - Graphic values for *Escherichia coli* in the grass pea miso sample (*Candida versatilis* and *Zygosaccharomyces rouxii*) with no fermentation stored at temperature of 37ºC (A), room temperature (B) and at a temperature of 4ºC (C).

(A)

Time (days)	**CFU/mL**
0	1,10E+05
2	0,00E+00
4	0,00E+00
7	0,00E+00
14	0,00E+00
30	0,00E+00

(B)

Time (days)	**CFU/mL**
0	1,10E+05
2	0,00E+00
4	0,00E+00
7	0,00E+00
14	0,00E+00
30	0,00E+00

(C)

Time (days)	**CFU/mL**
0	1,10E+05
2	2,50E+03
4	0,00E+00
7	0,00E+00
14	0,00E+00
30	0,00E+00

Listeria innocua

Table 7.3 - Graphic values for *Listeria innocua* in the grass pea miso sample (*Candida versatilis* and *Zygosaccharomyces rouxii*) with no fermentation stored at a temperature of 37ºC (A), room temperature (B) and at a temperature of 4ºC (C).

(A)

Time (days)	**CFU/mL**
0	**1,28E+05**
2	**0,00E+00**
4	**0,00E+00**
7	**0,00E+00**
14	**0,00E+00**
30	**0,00E+00**

(B)

Time (days)	**CFU/mL**
0	1,28E+05
2	0,00E+00
4	0,00E+00
7	0,00E+00
14	0,00E+00
30	0,00E+00

(C)

Time (days)	**CFU/mL**
0	1,28E+05
2	0,00E+00
4	0,00E+00
7	0,00E+00
14	0,00E+00
30	0,00E+00

Salmonella enterica Typhimurium

Table 7.4 - Graphic values for *Salmonella enterica Typhimurium* in the grass pea miso sample (*Candida versatilis* and *Zygosaccharomyces rouxii*) with no fermentation stored at a temperature of 37ºC (A), room temperature (B) and a temperature of 4ºC (C).

(A)

Time (days)	CFU/mL
0	1,01E+06
2	0,00E+00
4	0,00E+00
7	0,00E+00
14	0,00E+00
30	0,00E+00

(B)

Time (days)	CFU/mL
0	1,01E+06
2	0,00E+00
4	0,00E+00
7	0,00E+00
14	0,00E+00
30	0,00E+00

(C)

Time (days)	CFU/mL
0	1,01E+06
2	2,92E+04
4	1,50E+02
14	2,00E+01
30	0,00E+00

Staphylococcus aureus

Table 7.5 - Graphic values for *Staphylococcus aureus* in the grass pea miso sample (*Candida versatilis* and *Zygosaccharomyces rouxii*) with no fermentation stored at a temperature of 37ºC (A), room temperature (B) and at a temperature of 4ºC (C).

(A)

Time (days)	CFU/mL
0	4,67E+04
2	0,00E+00
4	0,00E+00
7	0,00E+00
14	0,00E+00
30	0,00E+00

(B)

Time (days)	CFU/mL
0	4,67E+04
2	0,00E+00
4	0,00E+00
7	0,00E+00
14	0,00E+00
30	0,00E+00

(C)

Time (days)	CFU/mL
0	4,67E+04
2	1,00E+03
4	0,00E+00
7	0,00E+00
14	0,00E+00
30	0,00E+00

Appendix 2 – Graphic values of grass pea miso sample (*Candida versatilis* and *Zygosaccharomyces rouxii*) with 7 months of fermentation for each pathogenic microorganism.

Bacillus cereus

Table 7.6 - Graphic values for *Bacillus cereus* in the grass pea miso sample (*Candida versatilis* and *Zygosaccharomyces rouxii*) with 7 months of fermentation stored at a temperature of 37ºC (A), room temperature (B) and at a temperature of 4ºC (C).

(A)

Time (days)	CFU/mL
0	4,88E+07
2	2,75E+03
4	7,65E+02
7	7,15E+02
14	6,90E+02
21	3,60E+02
30	1,45E+02
60	3,00E+01

(B)

Time (days)	CFU/mL
0	4,88E+07
2	1,31E+03
4	1,25E+03
7	7,05E+02
14	5,10E+02
21	5,60E+02
30	6,35E+02
60	3,60E+02

(C)

Time (days)	CFU/mL
0	4,88E+07
2	6,75E+02
4	1,23E+03
7	6,40E+02
14	6,70E+02
21	5,20E+02
30	6,50E+02
60	6,60E+02

Escherichia coli

Table 7.7 - Graphic values for *Escherichia coli* in the grass pea miso sample (*Candida versatilis* and *Zygosaccharomyces rouxii*) with 7 months of fermentation stored at temperature of 37ºC (A), room temperature (B) and at a temperature of 4ºC (C).

(A)

Time (days)	CFU/mL
0	2,55E+05
2	0,00E+00
30	0,00E+00
60	0,00E+00

(B)

Time (days)	CFU/mL
0	2,55E+05
2	0,00E+00
7	0,00E+00
30	0,00E+00
60	0,00E+00

(C)

Time (days)	CFU/mL
0	2,55E+05
2	4,25E+04
4	4,00E+04
7	1,50E+03
14	0,00E+00
21	0,00E+00
30	0,00E+00
60	0,00E+00

Listeria innocua

Table 7.8 - Graphic values for *Listeria innocua* in the grass pea miso sample (*Candida versatilis* and *Zygosaccharomyces rouxii*) with 7 months of fermentation stored at a temperature of 37ºC (A), room temperature (B) and at a temperature of 4ºC (C).

(A)

Time (days)	CFU/mL
0	2,03E+05
2	0,00E+00
4	0,00E+00
7	0,00E+00
14	0,00E+00
30	0,00E+00
60	0,00E+00

(B)

Time (days)	CFU/mL
0	2,03E+05
2	0,00E+00
4	0,00E+00
7	0,00E+00
14	0,00E+00
30	0,00E+00
60	0,00E+00

(C)

Time (days)	CFU/mL
0	2,03E+05
2	0,00E+00
4	0,00E+00
7	0,00E+00
14	0,00E+00
30	0,00E+00
60	0,00E+00

Salmonella enterica Typhimurium

Table 7.9 - Graphic values for *Salmonella enterica Typhimurium* in the grass pea miso sample (*Candida versatilis* and *Zygosaccharomyces rouxii*) with 7 months of fermentation stored at a temperature of 37ºC (A), room temperature (B) and a temperature of 4ºC (C).

(A)

Time (days)	CFU/mL
0	2,26E+06
2	0,00E+00
14	0,00E+00
21	0,00E+00
30	0,00E+00
60	0,00E+00

(B)

Time (days)	CFU/mL
0	2,26E+06
2	0,00E+00
14	0,00E+00
21	0,00E+00
30	0,00E+00
60	0,00E+00

(C)

Time (days)	CFU/mL
0	2,26E+06
2	2,73E+05
4	1,27E+04
7	0,00E+00
14	0,00E+00
21	0,00E+00
30	0,00E+00
60	0,00E+00

Staphylococcus aureus

Table 7.10 - Graphic values for *Staphylococcus aureus* in the grass pea miso sample (*Candida versatilis* and *Zygosaccharomyces rouxii*) with 7 months of fermentation stored at a temperature of 37ºC (A), room temperature (B) and at a temperature of 4ºC (C).

(A)

Time (days)	CFU/mL
0	4,09E+07
2	0,00E+00
30	0,00E+00
60	0,00E+00

(B)

Time (days)	CFU/mL
0	4,09E+07
2	0,00E+00
30	0,00E+00
60	0,00E+00
14	0,00E+00
30	0,00E+00

(C)

Time (days)	CFU/mL
0	4,09E+07
2	1,60E+07
7	1,91E+05
21	1,79E+03
30	6,50E+02
60	0,00E+00