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MASTER THESIS

Microbial examination of raw and extruded products for the production of a vegetarian meat analogue

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Abstract

The aim of the EU project LikeMeat was to develop a novel extruded meat analogue. To ensure product hygiene and food safety nine different proteins, derived from plants, milk and egg, were analyzed before and after the cooking extrusion process. In detail, the following microbial criteria were analyzed: Total Viable Count, the Psychotropic Count, *Enterobacteriaceae*, Coliforms, Coagulase-positive staphylococci, bacilli with the main focus on *B. cereus*, *Pseudomonas aeruginosa*, *Clostridium* with the main focus on *Clostridium perfringens*, *Listeria* spp., *Salmonella* spp. and Yeast/Mold counts. The results of the protein powders showed variable levels of potential food spoilage microbes and endospore-forming bacteria, but no human pathogens were detected. After the cooking extrusion of different protein mixtures the microbial load of the products was analyzed. The results displayed that bacterial endo-spore formers are able to survive the applied cooking extrusion process.

Zusammenfassung

Das Ziel des EU Projekts „LikeMeat“ war die Herstellung eines neuartigen Fleischersatzes aus extrudiertem Proteinalternativen. Im Rahmen dieser Diplomarbeit wurden neun verschiedene Pflanzen-, Milch- und Eiproteine vor und nach der Extrusion auf ihre mikrobiologische Kontamination untersucht. Analysiert wurde die Gesamtkeimzahl, die psychotrope Keimzahl, Enterobakterien, Coliforme, Koagulase-positive Staphylokokken mit einem Focus auf *Staphylococcus aureus*, *Bacillus* mit einem Focus auf *Bacillus cereus*, *Pseudomonas aeruginosa*, Clostridien mit einem Focus auf *Cl. perfringens*, Listerien, Salmonellen und die Hefe/Schimmelkeimzahl. In manchen Proteinpulvern wurde eine erhöhte Keimzahl an potentiellen Verderbserregern gefunden, jedoch wurden keine humanpathogenen Mikroorganismen nachgewiesen. Nach der Extrusion verschiedener Proteinmischungen wurden diese erneut mikrobiologisch untersucht. Es zeigte sich, dass bakterielle Endosporenbildner in der Lage sind den angewandten Kochextrusionsprozess zu überlegen.

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°C	degree Celsius
API 20E	Analytical Profile Index 20 tests for Enterobacteria
ASA	American Soybean Association
a_w	Water Activity
B.	*Bacillus*
BAC	*Bacillus* spp.
BC	*Bacillus cereus*
BOKU	University of Natural Resources and Life Science, Vienna
BP	Baird Parker Agar for *Staphylococcus aureus*
BSE	Bovine Spongiform encephalopathy
C.	*Clostridium*
CD	Celiac disease
CFC	Cetrimid Fucidin Cephaloridin Agar
CFU	colony forming units
CO_2	Carbon dioxide
CP	*Clostridium perfringens*
DGHM	German Association for Hygiene and Microbiology
DIN	German Institute for Standardization
DNA	Desoxyribonuclein acid
dNTPs	Desoxyribonucleosid triphosphates
DRBC	Dichloran Rose Bengal Red Chloramphenicol Agar
DRCM	Differential Reinforced Clostridial Medium
E.	*Escherichia*
e.g.	exempli gratia (latin) → for example
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorragic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EN	English
EPEC	Enteropathogenic *Escherichia coli*
et al.	et alia (latin) → and others
ETEC	Enterotoxic *Escherichia coli*
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration for the US
FRASER	Selective enrichment broth for Listeria, Fraser & Sperber (1988)
GRAS	Generally recognized as safe
h	hour, 1hour = 3600 s
HACCP	Hazard Analysis and Critical Control Points
HQ dist.	distilled water
HUS	Haemolytic uremic syndrome
H_2O	Hydrogen Oxide → water
H_2S	Hydrogen sulfide
ISO	International Organization for Standardization
IYP	International year of the potato
kg	kilo grams = 10^3 g
L.	Listeria
mA	milli Ampere = 10^{-3} Ampere
Mg^{2+}	Magnesium cation
min	Minute = 60 s
MKTTn	Muller-Kaufmann Tetrathionat Novobiocin Bouillon
mL	milliliter = 10^{-6} m^3

MRS	de Man, Rogosa & Sharpi agar
MYP	Mannitol Egg Yolk Polymyxin Agar
µ	mikro = 10^{-6}
NH_3	Azane → Ammonia
OCLA	Oxoid chromogenic Listeria Agar
PALCAM	Polymyxin, Acriflavine, Lithium chloride, ceftazidime, Aesculin, Mannitol
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PEMBA	Polymyxin, Egg Yolk, Mannitol, Bromothymol Blue Agar
RNA	Ribonuclic Acid
RNAse	Ribonuclease
rRNA	ribosomal Ribonuclic Acid
RVS	Rappaport-Vassiliadis Bouillon
S	Svedberg = 10^{-13} s to settle under centrifugal force
spp.	Species pluralis (latin) → multiple species
ssp.	Subspecies
Staph.	Staphylococcus
Taq	*Thermus aquaticus*
TBC	total bacterial count
TE	Tris-EDTA
TSC	Tryptose sulfite cycloserine agar
TSYEA	Tryptone Soy yeast extract agar
Tris	Trishydroxymethylaminomethane $(HOCH_2)_3CNH_2$
UV	Ultra violet light, Wellenlänge λ = 100 – 380 nm
UN	United Nations
UNFPA	United Nations Population Fund
US	United States of America
VRB	Violet red bile Agar
VRBG	Violet red bile glucose Agar
XLD	Xylose-Lysin-Desoxycholat Agar
YGC	Yeast glucose chloramphenicol Agar

1. Introduction

1.1. The LikeMeat Project – an overview

Many environmental and health organizations proclaim the shift from animal protein to a vegetable based diet (Sadler 2004). Although many new products in the meat analogue sector were released to the market in the last years, consumer acceptance is still very low, mainly due to sensory off-flavors, like soybean aftertaste or poor textural quality. So “the project LikeMeat aims at the development of meat analogues with excellent, well-accepted texture, juiciness, appearance and aroma based on plant proteins […].” (Fig. 1 & 2; LikeMeat Project Concept, 2010) Our focus of the associated group at BOKU was to ensure the microbial safety of the products and raw materials and therefore improvement of shelf life of the finished product. The definition of the project was to control all the raw plant proteins and the first extruded product for all eventually harmful or product spoiling microbiota. Currently there is only limited knowledge about microbiota and spoilage in meat analogues and their shelf life. The primary source of contamination of the end product is raw ingredients or spicing, and the first adding of water in the production process, a basic microbial population might emerge and, at the end of hydration, ideal conditions for many microbes can occur (Filho et al., 2005). One has to have in mind, that for new products, there is a lack of knowledge about consumer preference in preparation of the product and that the consumer doesn´t know, how a spoiled product might look or smell like, thus being a risk of food poisoning. Different plant proteins are used, because just in the combination of the different protein structures of these proteins, a hopeful approach for mimicking the fibrous and juicy texture of meat, in opposition to the soft and hardly textured products available up until now, can be made. Over the last years the number of vegetarians and “flexitarians”, consumers who are reducing their meat consumption to only once or twice a week, has been increasing in the European Union. For an estimated 8 - 9 % of the Germans in 2012 (Vegetarierbund Deutschland, 2012), consumption of meat is no longer acceptable, making them to the European country with the most percentage of vegetarians, in comparison to just 0.6 % being vegetarians in 1983. In Austria,

an estimated 3.6% of the population call themselves vegetarians (Vegetarierbund Deutschland, 2012), but also here numbers are rising.

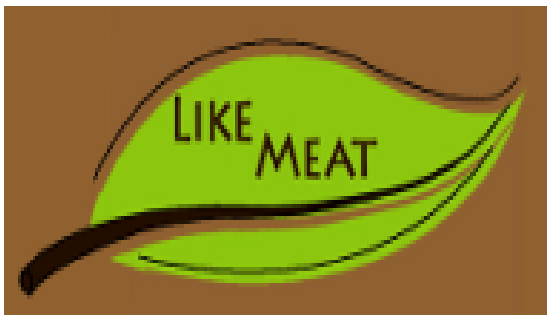


Figure 1: The LikeMeat logo

Figure 2: One of the most discussed pictures in promoting the LikeMeat project: The green Schnitzel by Standard Newspaper (2011)

1.2. The ecological impact of lifestock and meat production

Lifestock and meat production have a significant impact on nearly all aspects of the environment, from air, water and soil to biodiversity and climate. Yet the global demand for meat, milk and eggs is fast increasing, due to fundamental structural changes, such as rising incomes, urbanization and population growth, thus leading to extremely diverse claims on resources and nature (FAO, 2010a).

The lifestock sector occupies about 30 percent of the ice-free terrestrial surface through grazing and feedcrop production leading to pollution, emitting of nutrients and organic matter, pathogens and drug residues into bodies of water (Fig. 3). Including emitted gases such as carbon dioxide and methane livestock have a high impact on climate change and reduction of natural habitats. Carbon dioxide levels have increased by over 40 % over the past two centuries (NOAA, 2006). Today, CO_2 concentrations are higher than any time during the last 650 000 years (Siegenthaler et al., 2005) and methane concentration doubled since pre-industrial times (Spahni et al., 2005). As additional land available for cultivation is limited, most of the increase in production of feed crops will come from intensification of already cultivated land which can lead to degradation or at the expense of forests and other unspoiled landscapes. As one of the largest users of eatable crops, the meat production sector has to continue to improve the conversion of crops to animal

products. Besides us humans using the world´s renewable resources at rates that increasingly exceed their natural abilities to renew themselves (Westing et al., 2001). According to the Millennium Ecosystem Assessment (MEA, 2005), species are disappearing at 100 to 1000 times the normal background level. All these will limit natural resources and food which could, according to Renner (2002), lead to violent conflicts over the remaining resources and according to Schwartz and Randall (2003) could lead to droughts, famines and riots.

From an economical point of view, the rising incomes and the growing economies of developing countries, such as Asia, Latin America or the Near East, leads to a rising demand of products of animal origin and a shift of diets. While populations in developed countries are close to stagnant, nearly all population increase is occurring in developing countries. As the world population rose to 7 billion people this October (UNFPA, 2011) and is expected to rise to over 9 billion by 2050, the increase in livestock could lead to critical scarcity in all eatable products (Fig. 3; FAO, 2010b).

Fertility decline and an increasing life expectancy are leading to an overall ageing population globally, with the amount of people aged 60 or over will nearly triple by the middle of the century. Because age groups differ in their consumption patterns, animal protein consumption will rise, as adults and elderly people tend to eat larger amounts of meat and animal products than children (UN, 2005). Delgado et al. (1999) showed that as income grows, the expenditure on livestock products grows rapidly which will lead to a growing demand for these products.

A drastic change in dietary preferences occurs nowadays worldwide. Called the “nutrition transition” by Popkins et al. (2001), it is characterized by a shift from widespread undernourishment to richer and more varied diets and often overnutrition due to more pre-processed food, more food of animal origin, sugar, fat and often alcohol (FAO, 2006). In developing countries this shift, in contrast to developed countries, can occur within a single generation. The “nutrition transition” is driven by rising incomes and a decline of prices in real terms since the middle of the 20th century (Schmidhuber & Shetty, 2005). In

the developed countries there are recently concerns growing about the health, ethical and environmental impacts of an animal product and the livestocks welfare, building a class of “concerned consumers” (Harrington, 1994) which tend to reduce their consumption of livestock and go for certified products such as free range or organic foods (Krystallis & Arvanitoyannis, 2006).

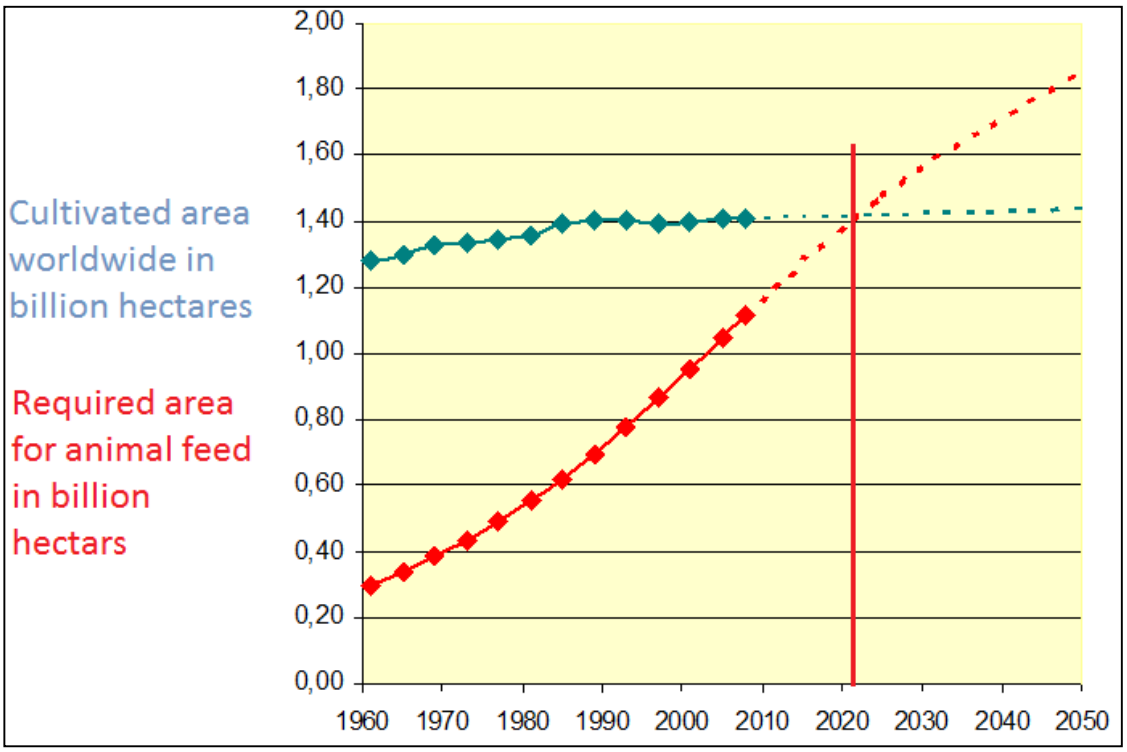


Figure 3: Developement of cultivated area to required area for animal feed. (FAO, 2010b)

The technological changes tend to a growing productivity in the lifestock sector, due to a widespread application of advanced breeding and feeding technologies, to irrigation and fertilization techniques in crop agriculture and to an improvement of post-harvest, distribution and marketing technologies (FAO, 2010a). The fastest growing subsectors in animal production are chicken and egg production, pork and dairy, mainly because of favorable feed conversion rates and thus leading to a lower price. The traditional way of feeding livestock on local feed resources that had no value as food for humans, such as crop wastes and browse, is replaced by feed concentrate. In 2002 roughly one-third of the global cereal harvest was used as livestock feed (FAO, 2005), and in 2007 almost 80% of agricultural land was used for grazing land

and cropland dedicated to feed production (FAO, 2009). More efficient breeds of livestock help reducing conversion ratios, shown by Arthur and Albers (2003), where conversion ratios for eggs in the USA have been reduced by approximately one-third in 40 years. Still the breeding industry has been less successful in developing breeds fitting in non-modified tropical environments, so enterprises will usually tend to control the environment around the animals, leading to higher use of antibiotics. Another trend in the livestock sector is the “industrialization” of livestock production. In the 1980s meat consumption in developing countries was less than 20 kg annually, making meat and animal products luxury and livestock was not only kept for food, but as power and manure supply, only butchered in case of emergency or special occasions. Since then meat consumption has at least doubled and total meat supply even tripled to 137 million tons (FAO, 2006) and there is a rapidly shift from ruminant animals such as cattle or goats to monogastric animals such as poultry and pigs. Poultry is by far the sector with the highest growth rates, on one hand because of the favourable feed conversion, but on the other hand because of the general acceptance by all major religious and cultural groups.

1.3. Meat alternatives

1.3.1. Market development and health benefits

Although meat analogues are relatively new to the Western Markets (Davies & Lightowler, 1998), meat protein replacements have gained interest in the last decade in Europe, due to severe plaques of animal farming such as BSE or Foot-and-mouth-disease (Morrison et al., 1999) and to an increasing interest in healthy and functional foods. In Asia, on the other side, products such as tofu or tempeh are eaten for centuries, whereas the launch of meat analogues in Europe started in the 1960s with texturized plant protein, produced usually by cooking extrusion of defatted soy meal or soy protein concentrates. Also different religious choices, like Islamic, Jewish or Hindi religion, may prohibit certain consumer groups from eating meat products. Meat is considered the highest quality protein source, containing all the essential amino acids closely resembling the human body and the functional properties exhibited by meat proteins cannot be replaced by any other food protein (Xiong, 2004). Their nutritional value is mainly due to their high biological value of proteins and their vitamins and mineral. Still, an excessive intake of meat products can´t be recommended because of their significant fat content, especially because of the high proportion of cholesterol and saturated fatty acids (Muguerza et al., 2004), which can lead to many diseases such as cardiovascular disease or cancer (Simopoulos, 2002). Plant products, on the other hand, have a high content of poly-unsaturated-fatty acids and especially α-linolenic acid, which is generally regarded as health (Jimenez-Colmenero, 2007). Another advantage of vegetable proteins is their low price in comparison to muscle protein (Singh et al., 2008). With more than 10% of all people on earth being malnourished (and especially protein – energy malnourished) (Myers, 2002), providing a safe, nutritious and wholesome food to a low price is a major challenge for the developing world (Bhat & Karim, 2009; Aiking, 2010).

1.3.2. Ingredients for vegetarian meat analogue

Soy protein

Soybeans (*Glycine max*) are leguminous plants related to clover, peas and alfalfa. They typically contain 35 to 40 % protein, making them the grain or legume with the highest amount of protein (Golbitz & Jordan, 2006). Soybeans are the single largest source for the manufacturing of texturized protein products worldwide (Riaz, 2004) and a number of raw materials made from whole soybeans, can be used in extrusion texturisation, like Soy protein isolate and soy protein concentrate. The utilization of soy food varies widely around the world. In East Asia soy beans are traditional food, as they are made to soymilk, tofu or fermented products (Fukushima, 1994). In the Western nations, soy beans in food applications and processing became popular after the U.S. Food and Drug Administration (FDA) approved the “Soy Protein Health Claim in 1999. In 2010 already 220.9 million tons were grown worldwide. They now have attracted people´s attention as an economic, healthy and high quality vegetable protein source. The American Heart Association even claimed Soy beans as beneficial to cardiovascular and overall health, due to their high content of polyunsaturated fats, fiber, vitamins and minerals and their low content of saturated fat (Sacks et al., 2006; Krauss et al., 2000). Also soybeans contain a well-balanced composition of amino acid and are rich in lysine, making them a good mix for cereal proteins (Sun et al., 2008). The major disadvantage of soy protein is the strong off flavor. On one hand, the bean and grass flavor, caused by lipoxigenase, on the other hand a bitter and astringent flavor caused by saponins and isoflavones (Okubo et al., 1992). Isoflavones and saponins, previously considered undesirable in the product, are now considered useful because of their anticarcinogenic activities (Fukushima, 2004).

Legume protein

Legumes are considered the poor man´s meat, because of their important place in diets of low income earners of developing countries (Serdaroglu et al., 2005; Oboh et al., 2009). In our project, Lupine protein (*Lupineus albus* L.) and pea protein (*Pisum sativum* L.) were taken. In many regions of the world,

legume seeds, with their 20 to 30 % protein in dry weight, are a unique supply of protein in the diet (Riascos et al., 2010), but they are relatively low in sulfur-containing amino acids when compared to animal protein. If they are combined with cereal protein, they provide a well-balanced amino acid profile (Boye et al., 2010) Furthermore legumes are a good source of minerals and B vitamins (Moussa et al., 1992). Unfortunately legume crops are common sources of food allergens (Singh & Bhalla, 2008; Riascos et al., 2010) and antrinutrients, such as protein inhibitors, lectins, polyphenol and phytic acid. The most characterized protein inhibitors are α-amylase inhibitors, which can cause a decrease in the postprandial blood glucose level, and trypsin inhibitors, which impair intestinal protein digestion. Cooking denaturates these enzyms so the unwelcome effects are usually just found when the seeds are consumed uncooked (Singh et al., 2008). Lectins in common beans (*Phaseolus vulgaris*) have sugar binding properties and hemagglutinating ability, causing gastroenteritis, diarrhea or nausea, and pose a possible health risk when eaten raw or incorrectly processed (Goldstein & Hayes, 1978). Polyphenols, such as tannins, can decrease the activity of digestive enzymes and amino acid availability but have high antioxidant activity (Salunkhe et al., 1982). Phytic acid is responsible for the reduction of the bioavailability of micronutriens, for example iron, tinn, potassium or copper, forming salts that are mainly excreted (Desphande & Cheryan, 1984; Crea et al., 2008). Thus can lead to mineral depletion and micronutrient deficiency (Thavarajah et al., 2009), but phytic acid may proof as an antioxidant or anti-cancer agent. Most of these anti-nutrients can be eliminated by chemical or physical treatments like cooking, dehulling, soaking or thermal treatments, for example polyphenols are significantly reduced by soaking and cooking.

Cereal protein

Cereals are the world´s most important food crop, making them the world´s most important food. Wheat has a protein content of 8 – 17.5 % and rice 7 – 10 % (Guerrieri, 2004), with an essential amino acid composition low in lysine (Singh et al., 2008). The most important economical by-product of the wet processing of wheat, rye or barley flour is gluten, making it an abundant plant protein source (Wang et al., 2006). It is unique in its ability to form, once it´s

plasticized, a cohesive blend with viscoelastic properties. Wheat gluten, divided into two subgroups the gliadins and glutenins, represents approximately 80 % of the protein contained in wheat. Wheat gluten is generally recognized as safe (GRAS), but can be harmful to individuals that carry Celiac disease (CD). CD is a disorder that is characterized by a permanent intolerance against gluten proteins (Green & Cellier, 2007). The T-cell lymphocytes in the small intestine respond to gluten with an inflammatory process, leading to intestinal damage, diarrhea, osteoporosis, iron deficiency anemia and other associated diseases (Sadler, 2004). CD occurs in about 1 % of the Western population (Green & Cellier, 2007) and the only therapy involves the lifelong elimination of gluten from the diet.

Milk protein

Agricultural milk is normally produced by cows (85 % of the world milk production). Normal bovine milk contains 30-35 g/L of protein, of which around 80 % is arranged in casein micelles. The other 20 % are normally summed up as whey proteins, where Lactoglobulin is the most common. Milk contains many antioxidant factors, like vitamins or enzymes. Studies suggest that bovine casein and whey protein are partly responsible for the poor bioavailability of iron in infant products, because of their binding to iron (Hurrel et al., 1989). An estimated 75 % of all human adolescents are lactose intolerant, so for usage of milk protein, measures of lactose residues in the protein have to be made.

Chicken Egg protein

Chicken eggs supply all essential amino acids for human nutrition. The egg white, which can be separated from the egg yolk, is often a by-product in the food industry. It contains of 90% water and about 10% protein, such as albumins, mucoproteins and globulins. Albumins or ovalbumins, the egg protein used in this sample, are water-soluble globular proteins. Allergic reactions to Chicken egg and Ovalbumin are common in infants and children and can cause severe allergic reactions. Most hen egg allergies, about 80% (Schäfer et al., 2007) tend to die down in children reaching school age. Denaturated ovalbumins are found to be not allergenic and therefore cooking

at temperatures over 80 °C will make it safe for consummation (Fehrmann, 2007).

Potato protein

Potatoes (*Solanum tuberosum*) are the world´s fourth largest food crop. Freshly harvested, a potato contains about 80 % dry matter and 16 % starch. A medium potato contains approximately 4g protein, which is often a byproduct in the potato starch industry. Finnish researchers showed that potato proteins can be capable from reducing high blood pressure (Pihlanto, 2008). Low allergenic and functional for emulsifying, foaming or gelation, it has also a potential for gut health and weight management. With a growing population FAO sees the potato as a mayor part in providing nutritious food for the poor and hungry. According to conservative estimations, the introduction of the potato in Europe and North America was responsible for a quarter of the World population growth between 1700 and 1900 (Nunn & Qian, 2011). Potatoes can grow more quickly, on less land and in harsher climates than any other major crop and up to 85 % of the plant is eatable in comparison to 50 % in cereal. In temperate climates, an irrigated hectare land can produce up to 35 tons of potato in 120 days of planting (IYP, 2008).

Mycoprotein

During the 1960s, nutritionists and politicians all over the world were concerned that the predicted growth in world´s human population would lead to a global protein shortage in the near future. After years of searching, a filamentous fungus (*Fusarium venenatum*) was found in 1967 in a field in Marlow, Buckinghamshire UK, which could be exploited by food scientists to produce protein for an ever growing mass of people (Denny et al., 2008). In order to bring this precious find to the market, scientists had to invest many years in researching the safety of the organism and the final product, but finally it was approved for sale in the United Kingdom in 1984 as “Quorn”(Quorn: Marlow foods, 2013). Mycoprotein is well tolerated by humans and has a very low allergenic potential (Miller & Dwyer, 2001). It contains dietary fibers, polyunsaturated fat and high-quality protein (Rodger, 2001), with a balanced essential amino acid composition. It is low in saturated fats and contains no

cholesterol, making it a weapon against high cholesterol levels in blood (Turnbull et al., 1990, 1992). Furthermore Mycoprotein is naturally low in sodium and a good source of zinc and selenium; just the levels of iron and vitamin B12 are low in comparison to meat (Denny et al., 2008).

Oilseeds

Oilseeds are different plants, normally grown for oil, making the protein a by-product. The level of proteins and especially the storage proteins of oilseeds are 13 – 17 % for safflower, 23 % for cottonseed and approximately 25 % for rapeseed. They make up for more than 20 % of world plant protein production, excluding soy (ASA, 2009). Fiber, phytic acid and phenolics are common in all oilseed plants but can be quite easily decreased by dehulling or other physical or chemical treatments (Prakash & Narasinga Rao, 1986; Arntfield, 2004).

1.3.3. Raw products for cooking extrusion

Traditional processing techniques for the production of plant protein, mostly soybean, remove and reduce undesirable components in the plant, like oligosaccharides or lipoxygenases. Extraction, heat treatment and centrifugation are used to separate the protein from other components. The plants are soaked and blanched in water, grinded and then filtered or centrifuged from fibers to produce dissolved protein concentrate. This product can be purified by ultrafiltration, to further lessen the concentration of undesirable components.

Protein Concentrate

To contain protein concentrate, the dissolved protein is precipitated or freeze dried. For Gluten, the protein is extracted through agglomeration. The traditional method to contain protein concentrate is to precipitate the filtrate. In Japan the original method for the production of tofu or bean curd is the coagulation of soybean milk. This resembles the production of cheese out to milk. Protein Concentrates normally contain 29 % to 89 % protein by weight.

Precipitation: Coagulation of the boiled protein filtrate with salt or acid is one of the most important steps in the production. Typically used coagulants for precipitation of plant protein are as per Guo & Ono (2005):

Salt coagulants: Chinese style tofu is normally produced with calcium sulfate or gypsum. This has marketing advantages, because the tofu can be sold as rich in dietary calcium. Japanese style tofu is traditionally produced with Nigari salts. These salts contain of a mixture of magnesium and calcium chloride. It produces a smoother tofu and the salt is higher soluble in water. Some old Japanese tofu producers use filtered sea water, which works too.

Acid coagulants: Glucono delta lactone is the most common acid coagulant. It is also used in cheese production for ovo-lacto vegetarians. It´s normally used together with calcium sulfate, to give soft tofu a smooth and tender texture. Acetic acid or citric acid could also be used.

Enzyme coagulants: In the production of cheese, the enzyme Lab from Calve stomach is traditionally used. For the production of vegetarian tofu papain or neutral proteases from microorganisms could be used too (Liu et al. 1997).

Crystallization through freeze drying: This method of production of protein concentrate is typically used for egg albumin. Known since the pre-Columbian Andean civilizations for the production of chuno, freeze-dried potatoes (Mahnke, 1982), the European food production uses this method since the Second World War, freezing the product with liquid nitrogen or dry ice instead of winter temperatures. After that the pressure is lowered to a few millibars and enough heat is supplied to sublime the water in the product. This method makes a protein concentrate dry and microbial stable which would otherwise be very moist and therefore would easily spoil (Harris & Angal 1989).

Extraction per agglomeration: Gluten is extracted from flour by mixing with water, kneading the mass till the gluten agglomerates and then washing out the starch. The dissolved starch can be reused by drying the filtrate. Approximately 65% of the water in the Gluten mass can be removed by a

screw press. The product is flour like powder with 7% moisture content (GEA Process Engineering 2013).

Spray drying: This method can be used for producing a dry product out of liquid slurry by drying with hot gas. It takes a liquid stream to a vapor and separates the solvent from the solute. The solute is then collected in a drum or cyclone (Niessen 2002).

Protein Isolate

Protein Isolates contain at least 90 % pure protein by weight. They are almost free of fat, carbohydrates or lactose. Most commonly two methods are used in the production (Berk, 1992):

Ion exchange: The ion exchanger extracts protein out of a floating stream of protein rich material according to their zeta-potential. It uses pH adjustments to activate and de-activate the attraction between the ion exchange resin and the specific protein. In the binding state non protein components are washed from the reactor. This process is highly specific to single proteins and therefore not all of the protein can be used from the material.

Membrane filtration: The membrane filtration normally used is a combination of microfiltration and ultrafiltration. Pressure is applied on a membrane which forces smaller molecules to go through the membrane while larger ones retrain. This method is not as specific as the Ion exchange method, but it will yield all the protein in the solution (de Wit, 2001).

1.3.4. Vegetarian products on the market

Traditional vegetarian meat alternatives

The difference in consumption of plant protein based foods between Western and Asian countries is described by D. Fukushima (1981): “In the U.S., the soy proteins are used as ingredients […]. In the Orient, however, soy proteins have been consumed for thousands of years, not as an ingredient, but as characteristic, unique soy protein foods.” Historically most of the traditional soy

protein foods originated from China, whereas today, Japan occupies the leading position in this field.

Tofu

Bean curd has been consumed in China for thousands of years with written record from the second century B.C. This typical Asian food is the basic ingredient for producing meat analogues on the basis of soy. Although it is rich in essential amino acids and considered one of the most healthy foods in the world, it hasn´t got the texture of meat. Still it can be smoked and seasoned, to archive a meat-like flavor (Wang, 1984). The production process starts with soy milk, which is boiled and then cooled to 75°C. A coagulant, normally gypsum powder or a solution of magnesium salts, is added and the curdling occurs. The supernatant whey is removed and the resulting curd is transferred to perforated boxes and pressed, much like in cheese preparation. When the curd is free from the remaining whey, the block is cut and wrapped for marketing. Fresh Tofu is stored, transported and sold refrigerated (Berk, 1992).

Texturized soy

The terms “texturization or texturing” applied to soy protein, mean the development of a physical structure which will provide, when eaten, a sensation of eating meat. At the first approach, the protein fibers where produced by a “spinning” process, quite similar to that used for the production of synthetic fibers for the textile industry. The starting material for that process is isolated soybean protein. The second approach converts the soy material into hydratable, chewy mass without true fibers. To produce such a mass there are two different processes: Thermoplastic extrusion and steam texturization. For these two processes products can be made from flour, concentrate or isolated protein. Texturized soy protein is already very common in Asian countries. Although the production process is very laborious, it has almost the same texture as meat and can be easily seasoned. The principal use of texturized soy protein is as a meat extender in comminuted meat products such as fillings, patties etc., where meat can be replaced up to 30 % with texturized soy protein without any loss of eating quality (Berk, 1992).

Seitan

Wheat gluten, which is known as Seitan in Asia, contains the proteins Gliadin and Glutenin. It is easy to prepare at household level, as one has to just rinse out the starch in normal wheat dough. This was also the first industrial process of production, known since 150 years as the Martin Process, but the problem with this system was the large amount of process water, which needed more than 15 parts of water to one of flour. The Raisio process (Dahlberg, 1977) works with the specific gravity of gluten and starch and separates them after hydration by centrifugation. It is the most cost effective and simple raw material for the production of meat analogues, such as f. e. sausages or burgers. Seitan has a fibrous structure, which is comparable to meat. As wheat is a major crop in almost all countries in the world, production is also possible on a regional level. Furthermore it can be minced and seasoned in almost every kind of way. But, as Celiac disease is on the rise, not all people can consume Seitan. Also there is a regular need for bacterial control, as the production of wheat gluten does not involve a heating or a decontaminating step (Sugden, 1997).

Tempeh

This Indonesian fermented food is produced by inoculating cooked soy beans with a *Rhizopus* mold. This mold produces a white soybean cake, which is very rich in protein and B vitamins, but is not fibrous. It has a kind of nutty taste and is very good for frying or as an inlay in soups. In a typical process of production, soybeans are soaked in water, dehulled and cooked for one hour. After that the soybeans are drained and air-dried. Tempeh from the previous day is used as a starter and the prepared soybeans are thoroughly mixed with it and let ferment for one to two days. The mass is bound together by the growth of the Rhizopus mycelium (Berk, 1992). Typically, Tempeh contains of 35% dry matter, which half of it protein. The traditional product is highly perishable and has to be consumed the day it is made. In industrial production, the product can be preserved by drying, freezing or blanching (Yung-Shung, 1981).

Producers of meat analogues available in Austria

Meatless

The Dutch processor manufactures a 100% vegetable fat free fiber, made from lupine, wheat or rice. They do not add artificial binder, colorizers or taste enhancers. They specialize on texturized vegetable protein to add to real meat or cheese products and for a substitution to vegetarian products (Fig. 4; Meatless, 2015).

Alnatura

Alnatura specializes in the production of hamburgers, made out of rice and potatoes, and sausages made from seitan and tofu. Their products are biologically grown, sustainably farmered and without additives or most of the most common allergic substances. Also they were voted Germany´s most sustainable company (Fig. 4; Alnatura, 2015).

Viana

Founded 1988 in Cologne, this producer concentrates on environmental protection and human health. Their main products are vegetarian products that resemble meat products, like Schnitzel, Chicken nuggets or filets or Cevapcici (Fig. 4; Viana, 2015).

Wheaty

Wheaty produces meat analogues on the basis of wheat gluten. Their products are lactose, milk, egg and palm oil free, produced biological and ecological and are totally vegan. The main products of this German producer are Sausages, Meat chunks for Döner meat or Gyros and Steaks (Fig. 4; Wheaty, 2015).

Spar-Veggie

Spar started with Spar Veggie an all vegetarian and vegan
line. They have over 30 different products, including sausages and burgers, with most of them on soy basis. The products are distributed at Spar Europe. They invented a soy tracking system, where you can look up through your production code, where the soybeans have been grown (Fig. 4; Spar, 2015).

Eden

Eden has a whole palette of cold-meat alternatives, like sausages, pâté and salami. Their products are on a soy and gluten basis, but they also have meat alternatives made from other crops, like rice or vegetables. Their philosophy claims to lessen their CO_2 emission and improve their sustainability (Fig. 4; Eden, 2015).

Valess

This company from the Netherlands produces vegetarian meat analogues on the basis of milk protein and plant fibers. Their product palette contains mostly breaded products like Schnitzel and Nuggets, but sausages, meatballs and filet too. Because of the milk, they are not suitable for vegans (Fig. 4; Valess, 2015).

Figure 4: Different logos of producers of meat analogues; 1: The meatless logo (Meatless, 2014); 2: The Alnatura logo (Alnatura, 2015); 3: The Viana logo (Viana 2015); 4: The Wheaty logo (Wheaty, 2015); 5: Spar Veggie logo (Spar 2015); 6: The Eden Germany logo (Eden, 2015); 7: The Valess logo (Valess, 2015)

1.4. Texturized proteins and meat analogues

Worldwide, the demand of more sophisticated meat alternatives grows and food manufacturers face the challenge of providing nutritious, economical and healthy foods, while ensuring that the product has an appealing taste and texture (Asgar et al., 2010). Texturized proteins contribute to two top food trends: High quality low fat foods and functional, nutraceutical foods (Riaz, 2004). They can be produced in various shapes and sizes and are accepted as vegetarian and halal foods (Lusas, 1996). Most common is texturized soy protein, usually processed by extruders to obtain a meat like structure. In 2007 approximately 1 million tons of texturized soy protein was produced worldwide (Hoogenkamp, 2007). Other raw materials have been used to produce texturized protein, such as wheat gluten, sunflower proteins or rapeseed proteins, which are till now only used in animal feed (Riaz, 2004). Texturized protein can be used in to different ways: Once as meat extender, which are not similar to meat in appearance and are mixed with meat for further processing, second as meat analogs. Analogs, which are produced to resemble meat in appearance, color, flavor and texture, represent the ultimate adoption of vegetable proteins (Singh et al., 2008). Acceptable texture and flavor often prove to be the biggest development challenges (Egbert & Borders, 2006). The addition of minor ingredients such as food flavor, color, pH modifier or emulsifier, increase the appearance and flavor of the meat analog (Riaz, 2004). Due to extrusion, meat analogs have a striated, layered structure, mimicking animal muscle.

The textural properties of freshly texturized proteins, like tensile strength, hardness, chewiness or shear stress can be analyzed by using a texture analyser (Chen et al., 2009). Lin et al. (2000) found out that the extrusion moisture content was significant to tensile strength, hardness, chewiness of the product and had a significant effect on the degree of texturization. It was shown that the products extruded with 60 % moisture content had the best fibrous structure. Cooking temperature had a significant effect on the tensile strength, but not on the hardness and chewiness.

1.5. High Moisture Extrusion Cooking

Extrusion cooking is a high temperature, short time thermal process that cooks, dries and restructures the product in one integrated operation (Fig. 5). Although the cost of installation and maintenance is high, the operation cost of cooking extruders and their low manning requirements have a significant reducing effect on the overall process cost (Nout et al., 1997). Wet extrusion of plant protein, at moisture contents above 40 %, is relatively less investigated compared to low or intermediate moisture extrusion. The first consideration of extruders as continuous bioreactors began, when Linko et al. (1983) began treating materials under elevated temperature, pressure and shear at moisture levels higher than 70 %. This made unconventional products such as texturized proteins possible. Texturization process can produce products "that imitate the texture, taste and appearance of meat or seafood with high nutritional value" (Cheftel et al., 1992). Lin et al. (2002) even states that moisture content is a more important factor than cooking temperature for both extrusion process parameters and product sensory characteristics. Feng et al. (2009) found out that higher moister reduced significantly the tensile strength, hardness, chewiness and the degree of aggregation. Some of these products, like the Japanese "Surimi", an extruded crab analog most often made from Alaska Pollack (*Theragra chalcogramma*) with egg white and starch (Shen & Wang, 1992) are already commercially available. During extrusion, protein structures are disrupted and altered under high shear, pressure and temperature (Harper, 1981). Protein cross-linking reactions occur as solubility decreases. Protein reactions, such as non-covalent, hydrophobic or disulfide bonds are formed upon cooling (Areas, 1992). The sharpest decrease of protein solubility occurs in the intermediate section of the extruder barrel, indicating formation of new disulfide bounds during the stage of dramatic increase in temperature and moisture (Liu et al., 2008). Different types of ingredients can enhance or inhibit the formation of fibers, for example it can be enhanced by the addition of different polysaccharids, such as starch or maltodextrins into the protein mix before the extrusion cooking, but the present of oil will decrease fibre formation, because of the lubricating effect of lipids. Furthermore the properties of protein extrudates are strongly influenced by the

operation conditions, the pH value, the ionic strength (Renkema et al., 2002) or the length of the cooling die (Thiebaud et al., 1996). The increase of temperature from 140 to 180 °C results in a proportional decrease in disulfide linkages (Ares, 1992), but temperatures lower than 90 °C hinder the expansion and layer formation (Cheftel et al., 1992). The cooling die is an important component of the extruder. It not only stabilizes the flow coming out of the barrel but also shapes the dough into a desirable product (Akdogan et al., 1997). Furthermore it will increase the viscosity of the hot product and therefore contributing to the correct viscosity and fluidity (Noguchi, 1989), and allow the protein to form longitudinally oriented bubbles, that give the product a similar texture to the layered characteristics of meat (Harper, 1981). Early research on the effects of extrusion on enzymes and microorganisms was one by Linko et al. (1981). MacDonald et al. (2009) showed that extruded soy protein was equally nutritious as the unextruded one for animals, and therefore considered a useful method to generate high-quality protein foods. Especially

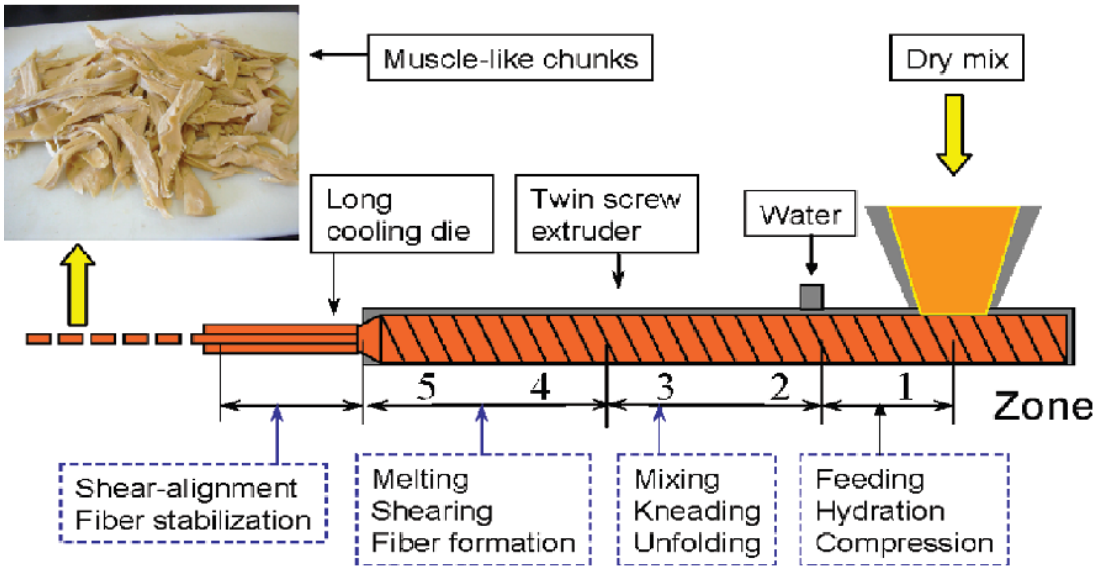


Figure 5: Scheme of a twin-screw extruder for high-moisture extrusion of protein rich materials into fibrous meat analogues (Liu & Hsieh, 2008)

interesting is the fact, that high moisture extrusion cooking can inactivate spores of *Bacillus* or *Clostridium*. It also has been shown that allergens of buckwheat and soy flours can be partially destroyed by high shear using twin-screw extrusion cooking (Hayakawa, 1995). Furthermore a reduction of mycotoxins by 12 % for moist products through extrusion processing was shown at temperatures from 116 to 120 °C (Scudamore et al., 2004).

1.6. Microbiology in plant-derived proteins and non-meat animal protein

The estimated risk of becoming ill as a result of microbial contamination of food is 10^5 times higher than the risk from pesticide contamination (Bennett & Klinch, 2003). Food borne diseases range from mild gastrointestinal upsets to life-threatening conditions (e.g. Botulism from *Clostridium botulinum*). Food poisoning is for most healthy people just an unpleasant episode from which recovery is done after a few days, but some groups of people are particularly susceptible to more serious consequences of infections, such as elderly, infants or immunocompromised (e.g. Chemotherapy or HIV patients) (Adams & Moss, 2008).

For the food industry, the costs can be enormous and it is not unusual for a company to go bankrupt, even companies not directly involved can suffer, as people tend to associate the same product with the same problems, such as the beef industry after the BSE outbreak. Also companies and countries spent huge amounts of money to prevent a further outbreak of a food borne disease. As Stockinger (2011) showed that Germany spent over one billion Euros just on tests for BSE in the last 10 years, not including new safety equipment and else, and didn´t find any case in years.

The German association for Hygiene and Microbiology has published guidance and critical values for Tofu from Soy protein (Tab.1). Still these don´t include microorganisms such as *Clostridium*, *Listeria*, yeast or molds which can cause food spoilage, intoxication or are human pathogens (DGHM, 1994). Filho et al. (2005) shows, that there is quite a high load of yeasts, molds and anaerobic spore-forming bacteria in ground soy protein.

Mwangi (2008) could show, that wild type *Bacillus* spores where reduced by about 4 log cycles in an inoculated soy-wheat protein matrix after extrusion at 180°C. But it made a great difference if extrusion was held at 180°C or at 130°C. Another very crucial point in production hygiene is the handling of the product after extrusion to prevent recontamination from raw product or personnel, where Enterobacteria, where especially *Escherichia coli*, yeasts

and molds are the main objective. Different seasoning for the end product could be a risk too, especially with highly pathogenic germs like *Salmonella* or *Listeria* germs, which can lead to food poisoning with only one living cell. The plan is that the final product will most likely be used in chilled convenience products, which will be post processed for extended shelf life. All these steps should lead to a safe and durable meat analogue product, but still, due to all the potential hazards in the production line, the hygienic conditions have to be controlled strictly.

Table 1: Guidance and critical values for Tofu from Soy protein (CFU/g) DGHM, 1994/95; currently under revision

Guidance and critical values for Tofu from Soy protein (CFU/g)

Bacterial colony	Guidance value	Critical value
Aerobic mesophilic count	10	-
Salmonella	-	n. d. in 25 g
Staphylococcus aureus	10	10
Bacillus cereus	10	10
Enterobacteriaceae	10	10

Many dry plant powders, like cocoa, show high occurrence of thermoresitant spores, with the majority being *Bacillus subtilis* (Lima et al., 2010). Dormant spores of *Bacillus*, *Clostridum* and related species can survive for hundreds of years (Kennedy, 1994), largely because spore DNA is well protected against treatments with wet and dry heat, UV and γ-radiation and toxic chemicals that would rapidly kill growing bacteria (Setlow, 2006). Even the first process criteria ensuring the safety of foods was the 12-D process of the destruction of *Clostridium botulinum* spores in low acid canned foods, the so called “botulinum cook” by Esty & Meyer (1922) and one of the first published quantitative risk assessment was related to *Bacillus cereus* in pasteurized milk (Notermans et al., 1997). Spores in water exhibit no detectable metabolism of endogenous or exogenous compounds. It was confirmed that pressure and heat increase spore permeability for protons, thus making them more vulnerable to low pH values (Wuytack et al., 2001). Sporulation of *Bacillus* species is induced by starvation for carbon and/or nitrogen. Spore germination can be triggered by addition of specific nutrients, such as L-amino acids, D-sugars and purine nucleosides (Setlow et al., 2007).

2. Aim of the study

The aim of the study was to test the microbiological quality of extrudates from different protein sources. The examination focused on the detection of spore forming bacteria, such as Bacillus spp. Further the applicability of current microbiological methods was tested. The protein samples were tested on microbial contamination before and after the extrusion process. The examination included pathogen bacteria common in eggs and milk such as *Listeria* and *Salmonella*, and food poisoning bacteria, such as *St. aureus* and *B. cereus*.

Total viable count (TVC)

The Total viable count represents the total load of vegetative microorganisms in a given sample. It counts all viable microorganisms that grow aerobically on Plate Count Agar at 37 °C for 24 and 48 hours. This test represents the general hygiene condition of the sample (Roszak & Colwell, 1987).

Enterobacteria

Enterobacteria are Gram negative, facultative anaerobe, rod shaped bacteria. Most of them are obligate pathogen organisms habituated in the digestion system, like *Escherichia*, *Enterobacter* or *Citrobacter*, or are food poisoning like *Salmonella*, *Shigella* or some groups of *E. coli*. A total count of Enterobacteria gives a good estimation of the fecal contamination of the finished product (Neidhardt et al, 1974).

Coliform bacteria

Coliform bacteria give an even better estimation about the fecal contamination of the product then enterobacteria, because they live almost exclusively in the digestion system of vertebrates (Conway, 1995). As Coliform bacteria are not likely to survive the extrusion process, their count can give an estimation of the hygienic conditions after the extrusion.

Escherichia coli

Escherichia coli are Gram negative and obligate pathogen Enterobacteria. *E. coli* bacteria are a natural inhabitant of the human and animal digestive tract and for healthy adults most of the strains are considered harmless to the human body (Feng et al., 2002). Whereas outside of the human body, *E. coli* is considered an indicator for fecal contamination and can cause infections like Meningitis, Inflammation or Peritonitis in people with reduced health, such as children, sick or elderly. In 2010, there were about 150 cases of *E.coli* enteritis per week in Germany (Benzler, 2011), therefore considered a very common disease. The most common strains that can cause food poisoning through contaminated food are enteroinvasive *E. coli* (This strain can cause diarrhea), enterotoxic *E. coli* (This strain can produce toxins), enteropathogene *E. coli* (This strain can cause infant gastroenteritis) and enterohaemorragic *E. coli* (This strain can lead to haemolytic uremic syndrome which can even lead to death)(Levine et al.,1978).

Salmonella spp.

Salmonella are a peritichous flagellated genus of Enterobacteria, which can grow aerobe and anaerobe. In difference to other Enterobacteria, *Salmonella* can grow on Lactose. Salmonellosis can be split into Enteritic Salmonellosis and Salmonella of the Typhi - Paratyphi group (Murray 1999). Most of all Salmonellosis in humans are caused by the subspecies *Salmonella enterica enterica*, so that was our main focus. With 25 307 cases of Enteritic Salmonellosis in 2010 in Germany, it is one of the leading causes of food poisoning in Europe (Benzler, 2011). Enteritic *Salmonella* are most common found in the intestines of free living animals, such as insects, rodents of free flying birds. In free range keeping, poultry can be infected through excretions of these. Humans, as the end of the infectious chain, get infected through not well cooked poultry meat or eggs, as *Salmonella* has a temperature optimum between 7 and 48 °C, with the peak at 37 °C as the average temperature of vertebrate guts. *Salmonella* can be severely harmed through freezing but most often it´s only sublethal, which allows them to survive in the ground in winter

(Török et al., 1997). In our product, a carryover can be caused by excretions of free living animals in the crop fields and are considered insanitary.

Because even one *Salmonella* bacterium can cause Salmonellosis (Black et al, 1960), there is only a test recommended, if, or if not there is *Salmonella* in the product.

Bacillus cereus **and other** ***Bacillus*** **species**

Bacillus spp. are Gram-positive, rod-shaped bacteria, which can be obligate aerob or facultative anaerob. Naturally habituated in the ground, they are ubiquitous in nature. Under stressful conditions all *Bacillus* species produce oval shaped endospores, which can survive under high pressure and heat and allows the bacteria to stay dormant for a long amount of time. Because of their ubiquitous nature, they can infect all foods contaminated by soil or dust. Some species of *Bacillus*, like *B. cereus*, can produce two different kinds of enterotoxins (Schoeni et al., 2005).

One is classified as the diarrheal type, causing diarrhea and gastrointestinal pain. These types can be found in all kinds of food over the pH value of 5, with an incubation time of 8 to 16 hours after ingestion and symptoms for up to 24 hours. These enterotoxins are very unstable to heat and inactivated by all kinds of in-kitchen-use heating mechanisms (Granum & Lund, 1997). The symptoms can be easily confused with an intoxication of *C. perfringens*.

The other enterotoxin built by *B. cereus* is emetic and is usually found in not properly prepared and stored rice. This toxin causes symptoms after half an hour till 6 hours after ingestion and symptoms of vomiting and nausea up to 24 hours after the first symptoms. The toxin is heat and pH stable and can´t even be destroyed by autoclaving (Granum & Lund, 1997). The intoxication of the emetic type is difficult to distinguish from intoxication of *Staphylococcus aureus*.

Coagulase positive ***Staphylococcus aureus***

S. aureus are Gram positive cocci with a 0.8 to 1.2 µm diameter. They are facultative anaerobe and don’t build endospores. They can grow at

temperatures up to 46 °C with an optimum between 35 °C and 37 °C. *S. aureus* are very resistant against many kinds of disinfectant and are one of the most common causes for sanies in humans (Benzler, 2011). Affection can lead from local inflammation to a sepsis and even to death in people with a debilitated immune system, like elderly or ill, thus being one of the leading causes of sudden hospital deaths (Bergdoll et al., 1981). Still *S. aureus* is found in 30 – 40 % of healthy people.

S. aureus can build up to six different enterotoxins of which five can cause food poisoning (Le Loir et al., 2005). All of them are very heat stable, even pasteurizing at 80 °C will affect them only after some hours. Enterotoxin A, the most common *S. aureus* toxin, is even stable to a change in pH or a_w value. Toxins are built in a temperature range from 10 to 45 °C, with an optimum at 37 to 40 °C.

Infections often start with an open wound, especially on hands, of a person preparing food, a fecal or airborne contamination (Lowy, 1998). All foods with a high protein or carbohydrate level are at risk, and as *S. aureus* can even infect farm animals, raw products are also in danger. Intoxication can start with an intake of 0.2 µg of enterotoxin A, produced by 10^6 cells per gramm food at 10 to 20 h at room temperature (Bergdoll et al, 1981). One to six hours after the intake of intoxicated food it can lead to diarrhea and sickness for up to two days.

Lactic acid bacteria

The group of Lactic Acid Bacteria includes many morphologically and taxonomically different microorganisms. They are obligate anaerobe and build mainly lactic acid, sometimes ethanol, CO_2 or acidic acid through fermentation. Commonly used in many food products, Lactic Acid Bacteria normally don´t cause harm when eaten. Here the main problem for our product is the building of many Off-flavors and gas, causing spoilage of the product or a non-acceptance of the consumer.

Yeast and molds

Yeast and molds are aerobe eukaryotic cells, classified as fungi. Popularly all fungi on food are called molds, independent of their systematic classification. Many family members of two of the most common molds, *Aspergillus* spp. and *Penicillium* spp., grow konidospores that are often in a bright green, blue or black color and are responsible for the bright spots on rotten food (Zipkes et al., 1981). Molds have a mycelium that consists of branching, thread-like hyphae, which have an approximate diameter of 5 to 10 µm. Molds need oxygen for the assembling of Ergosterin, a crucial substance to their cytoplasma membrane, which is why they tend to grow on the surface of foods (Schnürer & Jonsson, 1992). Most of them can grow under anaerob conditions, but are inhibited after a short time. Some molds can build mycotoxins which can cause food intoxication. Already in the middle Ages there was a mass intoxication called “St. Antony´s fire”, caused by the rye parasite *Claviceps purpurea* or ergot fungi (Mantle & Nisbet, 1976). It can cause hallucinations, muscle contractions and the die off of hands and feet. In 1960, 100000 turkeys died due to the feeding of molded peanut flour, which led to the discovery of Aflatoxins, a mycotoxin build by *Asperguillus flavus* (Hesseltine et al., 1966). Since then, many other categories of mold were found that could build potentially harmful toxins, like ochratoxins, fursariumtoxins or patulin. Intoxication of foods can have many causes: e.g. molds on the food, processing of molded raw material (very crucial in humid years), feeding of molded feed to livestock and carry over to milk and meat products (Fink-Gremmels, 2008). The dose of toxin in foods is most of the time non-lethal, but can cause many chronic intoxications, like liver of kidney cancer, liver cirrhosis, necrotis or teratogenensis (Newberne, 1974). Prophylactic arrangements, like out-sorting of molded raw material, pasteurization or production of uninfected raw products, have to be made, because if they are already in the product, the mycotoxins are very hard to get rid of. The main reproduction mechanism of many common molds is asexual. Even out of a tiny bit of mycelium can grow another mold. They can reproduce sexually through spores, classified e.g. as ascospores or basidospores.

Yeasts or endomycetes are single cellular fungi with a diameter of 5 to 10 µm. They need oxygen to grow, but can normally change their biosynthesis to fermentation (e.g. *Saccharomyces cerevisiae*). They reproduce asexual through constriction of daughter cells from the mother cell, called pullulation. Yeasts can grow in a broad pH range (pH 3 → 11) and temperature range (-10 °C → 45 °C) (Van der Walt, 1984). Osmophilic yeast can grow in an environment with high concentrations of sugar. Although yeasts are normally not toxic to humans, they can cause spoilage of the product due to many different forms of fermentation, like the building of ethanol, CO_2 or different aromatic esters.

We didn't know if our product will have a water activity less or higher than 0.95, so we used both ISO Norms (21527-1 for products with a water activity higher than 0.95 and 21527-2 for products with a water activity less than 0.95)

***Clostridium* spp.**

Clostridia are Gram positive rods, with up to 10µm length. They live in an anaerob environment, like ground or sediment of waters. They form heat and draught resistant spores, which can even survive extrusion. They are killed by heating up to 121°C for 3 minutes (Sugiyama, 1951). Clostridia are not very resistant against other bacteria, but in a bacteria free environment, they can show high growth rates (Roberts et al., 1981). Some build toxins, which made them dangerous in early production of canned food and caused many deaths. Today, through to hurdle systems in food quality management, intoxication from Clostridia became rare, still they are one of the most potent toxin builders in all the fauna, so for new products it has to be made sure, that no living cell or endospore can survive the production process. Of this family, two species tend to be common food poisoners:

***Clostridium botulinum*:** *C. botulinum* has its natural habitat in ground, sediment of lakes and shores. In animal and vegetable foods they can build the most toxic exotoxin for humans, called the Botulinum toxin (a. k. BOTOX). For an anaerobe bacterium, they are relatively insensitive to oxygen. In Germany, there were 2010 two cases of food-induced botulism (Benzler, 2011).

Some strains are proteolytic and cause recognize-able rotting, due to H_2S or NH_3 synthesis. They are most common found in canned food, but also meat and fish products.

Some on the other hand are saccharolytic, cause hardly any recognize-able rotting, and are therefore even more dangerous to humans (Roberts et al., 1981). They can be found most commonly in meat and fish products. Most cases of botulism in Europe are due to homemade pickle products or vacuum packed fish. All *C. botulinum* strains have a growth range between 3 – 50 °C, so they can grow in cans almost everywhere.

***Clostridium perfringens*:** This *Clostridium* species is common in ground and guts of humans and animals. They are divided into 5 different types, whereas Type A is the only strain that can cause food intoxication (Angelotti et al., 1962). This strain can build many enzymatic active toxins, like Lecithinase, Collagenase or Desoxynuclase, which can be necrotic to human cells (Petit et al., 1999). Also they can build a heat unstable enterotoxin, which is the main cause of *C. perfringens* food poisoning. *C. perfringens* has a growth range between 15 – 50 °C, with an optimum at about 44 °C. Usual levels of *C. perfringens* in the human body are up to 10^4/g feces, in the intoxicated state up to 10^6/g.

Pseudomonas aeruginosa

Pseudomonas spp. are obligate aerobe, gram negative rods with polar flagella. *Pseudomonas aeruginosa* is the most important species for food hygienic. It´s common in soil, surface water and on plants, and can therefore be found in vegetables, fruits and products out of these. It causes gangrene in humans, but for our case its most problematic feature is the building of proteolytic enzymes, which would cause spoilage in our product. *Pseudomonas aeruginosa* is very resistant against changing environmental conditions, and can survive and reproduce in almost every humid place, causing biofilms (Mann & Wozniak, 2012). These biofilms have a high potential of food intoxication, because they can provide home to many hazardous microorganisms like *Listeria*, or *Salmonella*.

Listeria monocytogenes

Listeria are short, Gram positive rods, that tend to form strings. They are facultative anaerobe and don´t build endospores. *L. monocytogenes* is psychotropic, can grow an environment from 0 °C (e.g. the fridge) to 44 °C and have generally slow growing rates and are relatively unstable to heat (Walker et al., 1990). *L. monocytogenes* can grow from a pH value of 4.5 to 9.0 (Buchanan et al., 1989). They are ubiquitous and can be found even in stool of healthy humans.

Contamination is common in raw meat (minced meat and poultry), fermented meat products, milk and milk products, in vacuum packed fish and raw vegetables. Farm animals can get infected through silage or mud. Food can be contaminated through infected people or non-sterile process equipment or working cloths. There are 377 reported cases of Listeriosis in Germany per 2010 (Benzler, 2011). Listeriosis has a mortality rate of 24 %, mainly among pregnant women, their fetuses or immunocompromised people (Farber & Peterkin, 1991). *L. monocytogenes* can cause infections with 100 germs /g, but valid data is not available so there is a no tolerance policy concerning *L. monocytogenes* in processed food.

Shelf life and Storage

Many microorganisms, for example *B. cereus, B. perfringens* or *Cl. botulinum*, can grow in temperatures of a household fridge. These psychotroph bacteria will spoil the product during shelf life and can be a major treat if they survive to the safety and storability of the product after the extrusion step. Toxin producing bacteria like B. cereus are another treat, as they can produce toxin even in low viable numbers.

Spoilage

As the product will be new to customers, they have no idea how a spoilt product will look or taste. Therefore, it is very important to examine which bacteria could be in the product to construct a method to show people the spoilage, for example a change in color of the packaging if the pH rises/drops. Another aspect is the use of spices and flavor that could recontaminate the extruded

product with viable bacteria or spores, or the general handling after the extrusion step, for example when packaging the product. Most products that are on the market are packed in anaerobe conditions, which will be beneficial to the growth of *Clostridium* spp. and other anaerobe bacteria like yeast or lactic acid bacteria, that can cause off flavors or sickness.

Goals

The goal was to check for and find all the microorganisms that are harmful to humans or cause spoilage in our product. Another goal was to examine which of the found bacteria can survive the extrusion process and therefore making the product a microbial safe and stable product to consume.

3. Materials and Methods

3.1. Plant protein samples

3.1.1. Raw protein powder

The raw products were delivered in glass and plastic bottles without chilling. At sample receipt the bottles were checked optically for correct packaging and photographed (Fig. 6) for documentation purposes.

First Lot 27.1. 2011

Pea Protein Isolate, Pisane M9 (PI)

Protein Isolate from yellow peas; Cosucra Groupe Warcoing S.A., Belgium
Lot number PPHM101620; Expiration date 25.4.12

Lupine Protein Concentrate Lupidor HP (LC)

A fraction of Lupinee meal; Hochdorf Nutrifood AG, Siedereistrasse 9, CH-6281 Hochdorf; Lot number 5.11.1.9_5393; No expiration date

Lupine Protein Isolate Lupi Typ E (LI)

Fraunhofer Institute, Giggenhauser Straße 35, D-85354 Freising
No Lot number, No Expiration date

Potato Protein Isolate Wiberg (KI)

Solanic B.V., P.O.Box 15; NL-9640 Veendam
Lot number 55033279; No Expiration date

Whey Protein Isolate Provon 190 (WI)

Glanbia Nutritionals; Carlow Road, Kilkenny Ireland
Lot number: 1007601_09873; Expiration date: 07.04.2009

Milk Protein Concentrate Megglosat HP (MC)

Meggle, Megglestrasse 5 – 12, D-83512 Wasserburg
No Lot number; No Expiration date

Soy Protein Concentrate Alpha 10 (SC)

The Solae Company North America; 1034 Danforth Drive 5c; US – MO 63102 St. Louis; No Lot number; No expiration date

Wheat Gluten vital - Kröner Stärke (WG1)

Hermann Kröner GmbH & Co. KG; PF 1354, D-49463 Ibbenbüren

No Lot number; No expiration date

Rice Protein Concentrate Remy Pro N80 (RC)

Beneo-Remy NY, Remylaan 4; BE – 3018 Leuven- Wjgmaal;

Lot number: 951013; No expiration date

Second sample Lot 22.3.2011

Wheat gluten (WG2)

Beneo-Remy NY, Remylaan 4; BE – 3018 Leuven- Wjgmaal

No Lot number; No expiration date

Hen egg albumin (EA)

Spray dried, pasteurized hen egg albumen powder; NIVE – P.O.Box 113, NL- 8070 AC Nunspeet; No Lot number; No expiration date

Figure 6: Dry protein powder; 1: Pea Protein Isolate, Pisane M9; 2: Lupine Protein Concentrate, Lupidor HP; 3: Lupine Protein Isolate Lupi Typ E; 4: Potato Protein Isolate Wiberg; 5: Whey Protein Isolate Provon 190; 6: Milk Protein Concentrate Megglosat HP; 7: Soy protein Concentrate Alpha 10; 8: Wheat Gluten vital - Körner Stärke; 9: Rice Protein Concentrate Remy Pro N80; 10: Wheat Gluten Beneo Remy NY; 11: Hen egg albumin (a: Transportation bottle; b: raw protein powder). All pictures taken by myself.

3.1.2. Extruded samples

Extruded samples were supplied in two lots immediately after extrusion. The extrusion was done on a laboratory scale extruder, with a maximum flow through at 0.5 kg / hour (Tab. 2). Samples were transported within 24 hours, one frozen at – 80°C and one fresh and chilled at 1°C. At sample receipt, the lots were checked optically for correct packaging conditions (labeling, bag sealing and temperature) and photographed (Fig. 7). The fresh samples were immediately microbial tested and the frozen samples were stored until the next day and then tested.

Third sample Lot 30.03.2011, Gasteiger / Wild:

- Lupine extract Lupidor 100 % (LE)
- Pea protein isolate Pisane 50 % + Hen egg albumin 50 % (PHE)
- Milk protein Megglosat HP 100 % (ME)
- Pea protein Pisane mg 100 % (PE)
- Wheat gluten vital (Körner) 100 % (WE)

Fourth sample Lot 06.04.2011, Gasteiger / Wild:

- Rice protein concentration Remypro N80 100 % (RE)
- Pea protein 'Pisane M9' 50% + Whey protein 'Provon 190' 50% (PME)
- Wheat gluten Beneo 100 % (WGE)
- Soya 'Alpha 10' 100 % (SE)
- Pea protein 'Pisane M9' 30 % + Lupi EE 70 % (PLE)
- Potato protein + Wheat gluten (PWE)

Table 2: Technical data for extruded samples from Freising

3rd Sample Lot	Date of extrusion	Temperature profile (°C)	Feed (kg/h)	H_2O (mL/min)	Moisture (%)
LE	29.03.	30/60/80/130/135	0.45	4.5	42.38
PHE	29.03	30/40/60/130/135	0.4	7.5	*
ME	28.03.	30/60/80/130/135	0.4	8.0	58.09
PE	28.03.	30/60/80/130/135	0.4	9.0	*
WE	29.03.	30/60/80/130/135	0.4	6.0	51.47

4th Sample Lot	Date of extrusion	Temperature profile (°C)	Feed (kg/h)	H_2O (mL/min)	Moisture (%)
RE	04.04.	30/60/80/130/135	0.3	10.0	~67
PME	05.04.	30/60/80/130/135	0.5	8.1	53.25
WGE	04.04	30/60/80/130/135	0.4	6.0	51.47
SE	05.04	30/60/80/130/135	0.4	9.0	60.77
PLE	04.04.	30/60/80/130/135	0.4	7.5	55.02
PWE	04.04.	26/40/70/130/140	0.5	15	64.03

* Undisclosed

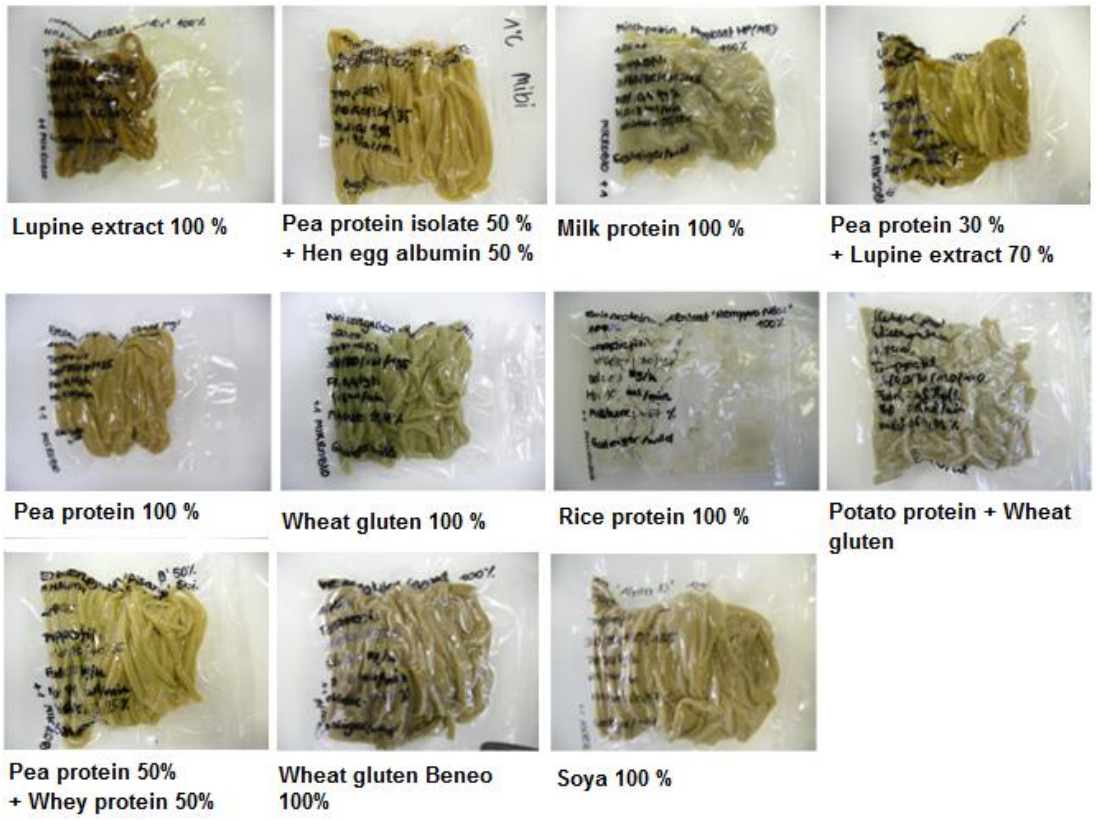


Figure 7: Samples of different protein extrudates at labscale

3.2. Examination of the microbial load

Methods for protein sampling

For dry plant protein:

Weight 10.0 g of dry plant protein into one Stomacher bag and fill with 90 mL buffered peptone water. Homogenize for 30 sec. Pour 1.0 mL of the undiluted solution with a sterilized pipette into an autoclaved 9 mL buffered peptone water sample tube to make a 1: 10 dilution. Take 1 mL from the 1: 10 dilution and pour it into another 9 mL buffered peptone water sample tube to make a 1: 100 dilution. Repeat to make a 1: 1000 dilution.

For extruded plant protein samples:

Weight 40 g of extruded plant protein sterile into 1 flame treated Laboratory mixer and mix for 1 – 3 sec. Then weight 10.0 g of the mixed sample into 1 Stomacher filter bag and fill with 90 mL buffered peptone water. Continue as shown for dry plant protein.

All cultures that could be positive for *Staphylococcus aureus, Clostidium* spp., *Pseudomonas aeruginosa*, *Bacillus* spp. and Pathogenes, are used for Cryocultures and DNA-Sequencing.

Table 4 and 5 give an overview of the used material and the ISO references. We did all testing regarding to ISO except for sampling. Most of the changes were necessary, because the material was hygroscopic or it was expected that the bacteria was inside the non-soluble material. Furthermore, we did some additional testing with newly developed material, for example different chromogenic media, and testing according to DIN norms, for example testing of *Bacillus* spp. with PEMBA.

Table 3: Material for sampling and enumeration of bacteria

Microbial Criteria	Material	Preparation and sampling
Sample preperation	Buffered peptone water (Oxoid) CM0509	Regarding to ISO 6887
	Ethanol absolute (Merck) K39539960851	For use in flame treatment
	Stomach lab mixer	For homogenization of samples
Total viable count	Plate count agar (Merck) 105463	Regarding to EN ISO 4833-6: 2003
Psychotrophic microorganisms	Plate count agar (Merck) 105463	Regarding to ISO 17410:2001
Yeast and moulds	YGC Agar (Merck) VM035900905	Regarding to ISO 21527-2:2008
	DRBC Agar (Oxoid) CM 0727	Regarding to ISO 21527-1:2008
	Chloramphenicol Selective Supplement (Oxoid) SR 0078E	Regarding to ISO 21527-1:2008
Mesophilic lactic acid bacteria	MRS Agar (Oxoid) CM 0361	Regarding to ISO 15214:1998
Enterobacteriaceae	Violet red bile glucose Agar (Oxoid) 857974	Regarding to ISO 21528-2:2004 and there is another reading of plates at 48 hours.
Coliforms	Violet red bile Agar (Lab M) 099390/261	Regarding to ISO 4832:2006 and there is another reading of plates at 48 hours.
Escherichia coli	Chromocult® Coliform Agar ES (Merck) 1.00850.0500	Regarding to Merck Lab manuals, bluish-violet colonies are counted
Salmonella spp.	Rappaport-Vassiliadis Bouillon RVS (Oxoid) CM 0866	Regarding to ISO 6579
	Muller-Kaufmann Tetrathionat Novobiocin Bouillon MKTTn (Biomerieux) 42114	Regarding to ISO 6579
Salmonella spp.	Xylose-Lysin-Desoxycholat-Agar XLD (Merck)	Regarding to ISO 6579
	ChromID *Salmonella* (Biomerieux) 43621	Regarding to ISO 6579

Microbial Criteria	Material	Preparation and sampling
Listeria monocytogenes	FRASER *Listeria* Selective Enrichment Broth base (Merck) 110398	Regarding to ISO 11290-1:2004
	FRASER Supplement (Oxoid) SR 0156	Regarding to ISO 11290-1:2004
	Half FRASER Supplement (Oxoid) SR 0166	Regarding to ISO 11290-1:2004
	Palcam *Listeria* Selective Agar Base (Oxoid) CM0877	Regarding to ISO 11290-1:2004
	Palcam *Listeria* Selective Supplement (Merck) 112122	Regarding to ISO 11290-1:2004
	Chromogenic *Listeria* Agar Base (Oxoid) CM1084	Regarding to ISO 11290-1:2004 In the ISO-Norm the medium is called OCLA
	Chromogenic *Listeria* Selective Supplement (Oxoid) SR0226	Regarding to ISO 11290-1:2004
	Chromogenic *Listeria* Differential Supplement (Oxoid) SR0244	Regarding to ISO 11290-1:2004
	Tryptone Soya yeast extract Agar (Fluka analytical) 93395	Regarding to ISO 11290-1:2004
	Hydrogene peroxide 3%	Regarding to ISO 11290-1:2004
	Blood agar Merckoplate® (Merck) 113414	Regarding to ISO 11290-1:2004
Bacillus cereus and other *Bacillus* spp.	Mannitol Egg Yolk Polymyxin Agar Base (Oxoid) CM0929	Regarding to EN ISO 7932_2004
	Polymyxin B Supplement (Oxoid) SR0099	Regarding to EN ISO 7932_2004 and DIN 10 198 part 1_1992
	Egg Yolk Emulsion (Fluka Analytical) 17148	Regarding to EN ISO 7932_2004 and DIN 10 198 part 1_1992
	Bacillus cereus selective Agar Base for PEMBA (Oxoid) CM0617	Regarding to DIN 10 198 part1_1992
	HiCrome™ *Bacillus* Agar (Fluka Analytical) 92325	Regarding to Sigma-aldrich Lab manual
	Brilliance™ *Bacillus cereus* Agar Base (Oxoid) CM1036	Regarding to Oxoid Lab manual

Microbial Criteria	Material	Preparation and sampling
Presumptive *Bacillus cereus* and other *Bacillus* spp.	Brilliance™ *Bacillus cereus* Selective Supplement (Oxoid) SR0230	Regarding to Oxoid Lab manual
	Blood Agar Merckoplate® (Merck) 113414	Regarding to Merck Lab manual
	Glucose-Tryptone Agar	5g Caseinpeptone, 0.75g yeast extract, 5g Glucose, 2.5 g NaCl, 7.5 mg Bromcresolpurpur, 6g Agar Agar, 0.5 L H_2O mixed and autoclaved for 15 min at 121 °C. Thermophilic *Bacillus* species grow in opak colonies with yellow halo
Clostridium spp.	DRCM (Merk) *Clostridium* Differential Broth, 38210000	Regarding to Merck Lab manual
	Paraffin, sterile filtrated	For anaerob covering
Clostridium perfringens	TSC Perfringens agar base (Merck) VM 722872701	Regarding to ISO 7937-2004
	Cycloserin	Regarding to ISO 7937-2004
Coagulase-positive *Staphylococci*	Baird Parker Agar (Oxoid) CM0275	Regarding to ISO 6887-1:2004
	Egg yolk tellurite emulsion (Oxoid) SR0054	Regarding to ISO 6887-1:2004
	Hi Chrome™ Aureus Agar Base (Fluka Analytical) 05662	Regarding to Sigma-aldrich Lab manual
	Staphylococcus aureus Monotec test kit (Fluka Analytical) GA 19457	Regarding to Sigma-aldrich Lab manual. For testing of Coagulase
Presumptive *Pseudomonas* spp.	*Pseudomonas* Agar Base (Oxoid) 925653	Regarding to ISO 13720
	Pseudomonas CFC Supplement (Oxoid) SR0103E 948180	Regarding to ISO 13720
	Oxidase Reagent	Regarding to ISO 13720
	Escherichia coli strain	Regarding to ISO 13720
	Oxidase positive *Pseudomonas* strain	Regarding to ISO 13720

Table 4: List of ISO-Norms

Microbial Criteria	Method	Description
Sample preperation	ISO 6887-4: 2003/2004	Microbiology of food and animal feeding stuffs – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products
Sample preperation	ISO 7218:2007	Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations
Total viable count	ISO 4833:2003	Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of microorganisms. Colony-count technique at 30°C
Psychotrophic microorganisms	ISO 17410:2001	Microbiologie of food and animal feeding stuffs –Horizontal methods for the enumeration of psychotrophic microorganisms.
Yeast and moulds	ISO 21527-1:2008	Microbiology of food an animal feeding stuffs – Horizontal method for the enumeration of yeast and moulds – Part 1: Colony count technique in products with water activity greater than 0.95
Yeast and moulds	ISO 21527-2:2008	Microbiology of food an animal feeding stuffs – Horizontal method for the enumeration of yeast and moulds - Part 2: Colony count technique in products with water activity less or equal 0.95
Mesophilic lactic acid bacteria	ISO 15214:1998	Microbiology of food an animal feeding stuffs – Horizontal method for the enumeration of mesophilic lactic acid bacteria – Colony-count technique at 30°C
Enterobacteriaceae	ISO 21528-2:2004	Microbiology of food an animal feeding stuffs – Horizontal method for the detection and enumeration of Enterobacteriaceae – Part 2: Colony-count method

Microbial Criteria	Method	Description
Coliforms	ISO 4832:2006	Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of Coliforms – Colony count technique
Salmonella spp.	ISO 6579:2002	Microbiology of food and animal feeding stuff – Horizontal methods for detection of *Salmonella* spp.
Listeria monocytogenes	ISO 11290-1:2004	Microbiology of food and animal feeding stuff – Horizontal methods for detection and enumeration of *Listeria monocytogenes*. Part 1: Detection methods
Presumptive *Bacillus cereus*	ISO 7932:2004	Microbiology of food and animal feeding stuff – Horizontal methods for enumeration of presumptive *Bacillus cereus* – Colony count technique at 30°C
Clostridium perfringens	ISO 7937-2004	Microbiology of food and animal feeding stuff – Horizontal methods for enumeration of *Clostridium perfringens* – Colony count techniques
Coagulase-positive *Staphylococci*	ISO 6888-1:1999	Microbiology of food and animal feeding stuff – Horizontal methods for enumeration of coagulase-positive *Staphylococci* (*Staphylococcus aureus* and other species). Part 1: Technique using Baird Parker agar medium
Presumptive *Pseudomonas* spp.	ISO 13720-2010	Meat and meat products – Enumeration of presumptive *Pseudomonas* spp.

3.3. Staining and Microscopy

GRAM STAINING

Gram staining (Gram 1884) is an easy method to distinguish between Gram positive and negative bacteria. Gram positive bacteria are blue – violet, Gram negative bacteria are red.

SPORE STAINING

This staining method of Ashby (1938) is a good verification of spore forming bacteria, like *Bacillus* or *Clostridium*. This method is possible, due to a higher fixation of dyes in the spores.

LIPID BODY STAINING

This staining method of Holbrook and Anderson (1980) combines the spore staining of Ashby (1938) and the intracellular lipid body staining of Burdon (1946). The staining can be used for further identification of *B. cereus*.

MATERIAL:

5 % (mass) Malachit green solution

0.3 % (mass) Sudan black solution in 70 % ethanol

Xylol

0.5 % (mass) Safranin solution

METHOD:

Air dry and heat fix a one-day culture on a microscope slide. Hold the microscope slide over boiling water and over float it with a 5 % Malachit green solution for two minutes, then flush it with distilled water and dry it with blotting paper. Color the microscope slide with 0.3 % Sudan black solution for 15 min. Wash the microscope slide with Xylol for 5 sec. and dry with blotting paper. Color the microscope slide with 0.5 % Safranin solution for 20 sec. Wash with distilled water, let dry and watch under microscope (1000 – 1500 enhancement). Lipid bodies are black, endospores are green and cytoplasm is red.

3.4. PCR and Electrophoresis

3.4.1. DNA Purification

Today the effect of purified DNA cannot be underestimated and is crucial for many applications. The basic steps of DNA isolation are disruption of cellular membranes and structures to create a cellular lysate, separation of the DNA from insoluble materials, like cell debris, and the purification of the DNA from soluble proteins. DNA-Purification is done before a PCR, to get a small volume of pure DNA to thermocyle with. The method we used is named “5 Prime Pure DNA Purification”.

MATERIAL:

- 5Prime Lytic Enzyme Solution – 650 µL; Ref # 2301420, Lot # 8273174
Store at 2-8°C, Exp: 2011-07-06

- RNase A 4 mg/mL

- 5Prime ArchivePure DNA; Yeast & Gram-+ Kit, Mat # 2900275 / 2301210, Lot # 821315; Store at 15-25°C, Exp: 2010-03

- 0.5 mL Gram-positive bacteria overnight culture
(expected Yield: 3-30 µg DNA)

METHOD:

For cell lysis, add 0.5 mL cell suspension (e.g. overnight culture containing 0.5-1.5 billion cells) to a 1.5 mL microfuge tube on ice and centrifuge at 13,000-16,000 x g for 5 to 60 seconds to pellet cells. Remove as much supernatant as possible using a pipette. Re-suspend cells with 300 µL cell suspension solution and add 1.5 µL Lytic Enzyme Solution. Invert the tube 25 times to mix and incubate at 37 °C for 30 min for cell wall digestion. While incubation, invert the sample occasionally. Centrifuge at 13,000-16,000 x g for 1 minute to pellet the cells and remove supernatant. Add 300 µL Cell Lysis Solution to the cell pellet and gently pipet up and down to lyse the cells.

For RNase Treatment add 1.5 µL RNase A Solution to the cell lysate and invert the tube 25 times to mix. Incubate for 15 – 60min at 37 °C.

Cool the sample to room temperature then add 100 µL Protein Precipitation Solution and vortex vigorously for 20 seconds. Centrifuge at 13,000-16,000 x g for 3 minutes till the precipitated proteins form a tight white pellet. If the protein pellet is not tight, repeat vortexing followed by incubation on ice for 5 minutes, then repeat centrifugation.

Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a clean 1.5 mL microfuge tube containing 300 µL 100% Isopropanol (2-propanol) and mix the sample by inverting gently 50 times. Centrifuge at 13,000-16,000 x g for 1 minute, till the DNA is visible as a small white pellet. Pour off the supernatant and drain tube briefly. Add 300 µL 70 % Ethanol and invert tube several times to wash DNA pellet. Centrifuge at 13,000-16,000 x g for 1 minute, then carefully pour off the ethanol. Invert and drain the tube on clean absorbent paper and allow air-drying 10-15 min.

Hydrate the DNA by adding 100 µL DNA Hydration Solution, to get a DNA concentration of 100 µg / mL. Incubate sample for 1 hour at 65°C and overnight at room temperature. Tab tube periodically to aid in dispersing the DNA. For short term storage, store DNA at 4°C. For long term storage, place sample at -20°C of -80°C.

3.4.2. 16S rRNA SPECIFIC PCR

The polymerase chain reaction (PCR) is a fast and easy way to replicate a single or a few copies of DNA pieces, to generate millions of copies of a special sequence (Saiki et al 1988). The method relies on cycles of repeated heating and cooling, thus allowing the heat stable DNA-Polymerase to anneal to the DNA strain, extend it with nucleotids, that are provided, and to denaturate after.

The 16S ribosomal RNA is a component of the 30S small subunit of prokaryotic ribosomal RNA. It is 1542 nucleotids in length and is used for exploring phylogenies in bacteria since 1977 (Woese and Fox 1977). In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species specific signature sequences, which can be useful for bacterial identification (Kolbert and Persing 1999).

A usual start mixture for PCR would contain of such (Sambrook and Russel 2001):

- **A DNA strain**, that contains the sequence that we want to amplify, in our case it´s the **16S rRNA of gram positive bacteria**
- **Two primers** that are complimentary to both of the 3´strands ends of the DNA sequency of the sample
- **Taq-Polymerase** or another Polymerase with the temperature optimum around 70 °C
- Desoxynucleosid triphosphates **(dNTPs)** as building blocks for the polymerase
- **Buffer solution** to provide optimum activity and stability for the DNA-Polymerase, containing divalent cations (e.g. Mg^{2+}) and potassium ions.

A PCR is normally carried out in small volumes (in our case 24.5 µL) in a small sample reaction tube in a thermocylcer (in our case the Eppendorf Mastercycler® eP gradient). The thermocyler heats and cools the sample according to the reaction steps necessary for the Polymerase and primer annealing.

MATERIAL:

- Eppendorf Mastercycler® eP Gradient No 534100129 (Fig. 41)

- CELLO-P6; Eurofins MWG Operon, 124 25-3484-6/6
Expiration date June 17th 2009

- CELLO-P0; Eurofins MWG Operon, 84 25-3484-5/6
Expiration date June 17th, 2009

- Premix MM, 22.5 mL

METHOD:

Run: 95 °C 30 seconds, 50 °C 30 seconds, 68 °C 3 minutes, 25 Runs

3.4.3. Agarose Gel Electrophoresis

MATERIAL:

- Water bath (T=50 °C)

- 2 L 0.75x TE-Buffer

The Buffer supplies ions like Mg^{2+} for the transmission of electricity during the electrophoresis.

- Ethidium bromide

Ethidium bromide works as a fluorescent tag for DNA based on the insertion of Ethidium bromide molecules into the planar bases of the DNA. Under UV Light Ethidium bromide is fluorescenting.

- Loading dye

Loading dye contains bromophenol blue and xylene cyanol for visual tracking of DNA migration during electrophoresis. The presence of glycerol ensures that the DNA in the ladder and sample forms a layer at the bottom of the well. The EDTA included in the solution binds divalent metal ions and inhibits metal-dependent nucleases.

- Marker

The Marker is a DNA Solution with defined fragments of DNA. It works as a method of size comparison as DNA fragments with equal length will travel the same way in an agarose gel electrophoresis.

METHOD:

Weight the calculated amount of agarose in a sterile flask and fill it up with 40 mL 0.75x TE-Buffer for a small gel. Mark the volume on the flask and let the agarose swell for 5 minutes and dissolve in a microwave oven. Fill the potential loss with sterilized water then cool down to 50 °C. Tape around the end of the gel tray and pour the warm gel solution without air bubbles in it. Insert an

electrophoresis comb into the gel tray, cover it and let it polymerize for half an hour. In the meantime prepare the electrophoresis chamber by pouring approx. 1.9 L of 0.75 x TE-Buffer into the electrophoresis chamber. Adjust the temperature to 20 °C and turn on the power supply.

After the gel is polymerized, remove the tape from the gel tray and put the gel tray with the gel in the electrophoresis chamber. Mix loading dye, PCR-product, and Marker by vortexer shortly. Prepare parafilm and pipette 1.5 µL loading dye and 5 µL PCR-product or Marker on the parafilm. Mix directly and load the mixed sample on the wells of the gel. Repeat for the other samples. Turn on the pump and set to ‘pump delay 4.5 min’. Set Voltage= 80(V); Currents= 2000 (mA); temperature= 20 (°C) and running time then close the lid and start the electrophoresis. Turn off the electrophoresis when the running time passes.

After putting on nitrile gloves, take the gel out from the electrophoresis. Fill 350 mL sterile water into a staining container and add 35 µL Ethidium bromide. Mix thoroughly and put the gel into the staining container. Staining will take for 30 min.

In the meantime prepare the second staining container with 350 mL running 0.75x TE-Buffer. Take the stained gel from the staining to the de-staining container.

Take the gel out of the destaining solution and put it into the Chemilmager and start the program. Press ‘Expose preview, turn on UV Light and choose the duration of exposure. Check the focus and zoom if necessary and press ‘acquire’. Save where desired.

3.5. Storage of bacterial isolates

Cryocultures are an easy way to ensure reproducible results and continuity in research. Serial subculturing can lead to contamination or genetic drift. Cryopreservation conserves genetically stabilized bacterial cells, and proteins or DNA are preserved unchanged. As water is the major component of all living cells, cellular processes stop when all water is converted to ice and thus, for all practical purpose, stops time for the cell. Ice forms at different rates during the cooling process. Slow Cooling forms less intracellular and more extracellular ice, thus leading to an osmotic imbalance, which can lead to cell death. Fast cooling can lead to too much intracellular ice and cell damage due to crystal formation (Simione, 1992). Also the cell permeability affects the rate of water loss, as it is shown that more permeable cells are able to tolerate rapid cooling better than less permeable cells (Nei et al., 1969). Most of the time, a cooling rate at 1°C / minute is shown to have the best effects. Also using cryoprotective agents, like Glycerol, can protect the cell during freezing. For microorganisms, generally, the greater the numbers of cells present initially, the greater the recovery. For most cells, that means concentrations of approximately 10^7 /mL, best grown in an over-night culture in cultural broth (Nalgene, 2006).

MATERIAL:

- Soy Tryptone Agar

- Culture Medium (Roth) 200157963

- Glycerol 87 % (Applied Chemistry) Charge 4T001658

METHOD:

Plate one single culture from differential medium to Soy Tryptone Agar and incubate for 24h at 37 °C. Take again one single culture and put it in 9 mL culture medium in a 15 mL tube. Incubate for another 24h at 37 °C, best on a shaker. After that infuse the probe with 2 mL glycerol 87 %, shake well and transfer to 1.5 mL Cryotubes. After that store in minus 20 °C store room.

3.6. pH-Measuring of extruded product

MATERIAL:

- Ringer Solution (2 x 1 L)

8.6 g/L Sodium Chloride,

0.3 g/L Potassium Chloride,

0.33 g/L Calcium Chloride

METHOD:

We did one measurement with HQ water and one measurement with Ringer solution. The extruded product was weighted into a stomacher bag and filled 1:10 with HQ water / Ringer solution. It was mixed in a stomacher for 30 seconds and pH-measured.

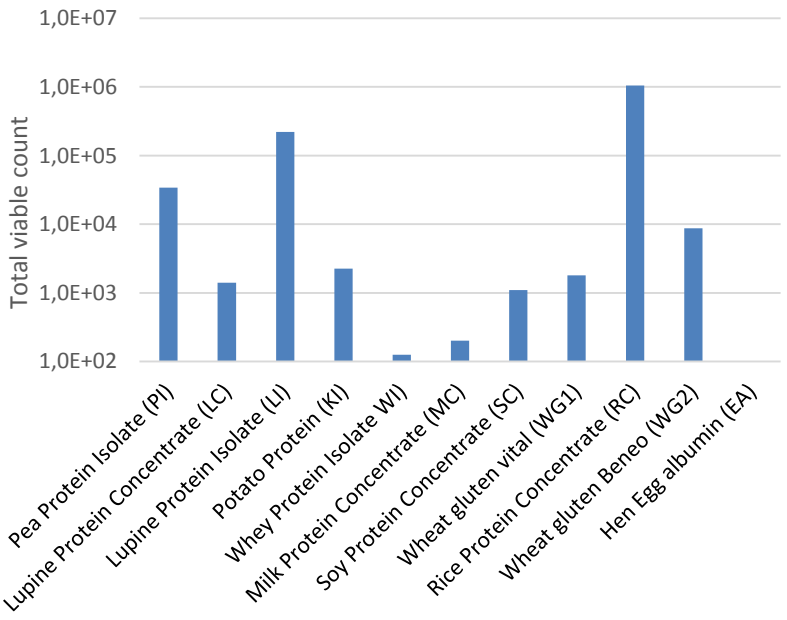
4. Results

4.1. Microbial examination of dry protein

Total Viable count

The dry protein showed a contamination with different microorganisms (Tab. 5). We found different strains of *Bacillus* spp., which sometimes overgrew the other microorganisms and could not be counted until high dilutions. Rice protein concentrate and Lupine protein isolate showed high values for overall microorganism, but both were under the guidance level. Hen Egg Albumin was under the detection limit of 100 CFU/g. The two protein samples from milk (Whey protein isolate and milk protein concentrate) showed very low levels of bacterial contamination and were almost under the detection limit. The other samples were between 1000 and 10000 CFU/g.

Table 5: Total Viable Count (CFU/g) in dry protein



Samp.	CFU/g
PI	$3.4*10^4$
LC	$1.4*10^3$
LI	$2.2*10^5$
KI	$2.25*10^3$
WI	$1.25*10^2$
MC	$2.0*10^2$
SC	$1.1*10^3$
WG1	$1.8*10^3$
RC	$1.05*10^6$
WG2	$8.75*10^3$
EA	<100

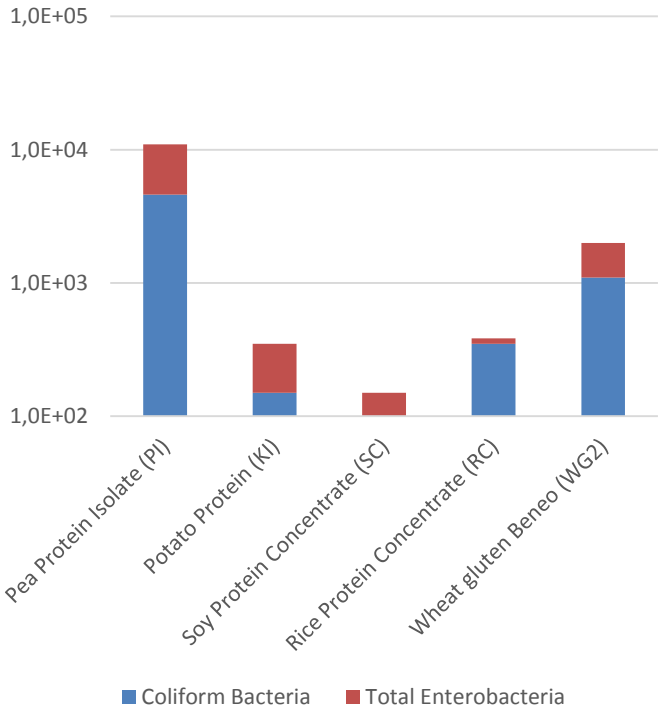
Enterobacteriaceae

Enterobacteria were in four of the eleven samples in significant concentrations (Tab. 6). In Lupine concentrate and isolate, whey isolate, milk concentrate, soy concentrate, hen egg albumin and wheat gluten from vital the concentrations were under the detection limit of 100 CFU/g or very low above it. In pea protein isolate the Enterobacterial count was above 10^4 CFU/g, with the Coliform count above 6000 CFU/g. The count for *Escherichia coli* was in all samples under the limit of detection at 1000 CFU/g.

Pathogenic bacteria

In none of the tested samples were either *Listeria monocytogena* or *Salmonella* spp. found.

Table 6: Enterobacteria, Coliform bacteria and *Escherichia coli* in dry protein (CFU/g)



Samp.	Entero-bacteria	Coliform Bacteria
PI	$1.1*10^4$	$4.6*10^3$
LC	<100	<100
LI	108	100
KI	$3.5*10^2$	$1.5*10^2$
WI	<100	<100
MC	<100	<100
SC	$1.5*10^2$	100
WG1	<100	100
RC	$3.85*10^2$	$3.5*10^2$
WG2	$2.0*10^3$	$1.1*10^3$
EA	<100	<100

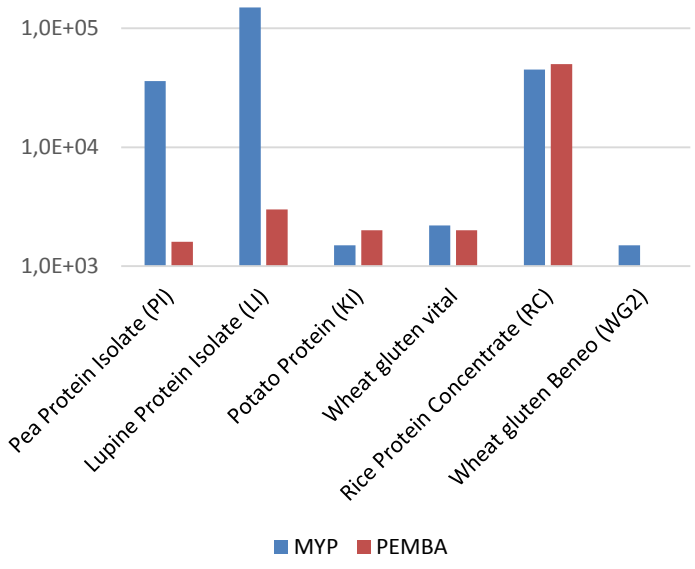
Bacillus spp.

Bacillus spp. were found in 6 of the 11 samples (Tab. 7), the other 5 samples were under the detection limit of 1000 CFU/g. In Rice protein concentrate *Bacillus cereus* was detected per DNA Sequencing. In 3 samples, Rice protein concentrate, Lupine Protein Isolate and Pea Protein Isolate the concentration of *Bacillus* spp. bacteria was over the guidance level of 10000 CFU/g. In Pea Protein Isolate and Lupine Protein Isolate the recovered Bacillus spp. in MYP and PEMBA Agar differed greatly, where with MYP Agar more *Bacillus* spp. were found.

Coagulase positive *Staphylococcus aureus*

In 9 of the 11 samples Staphylococcus levels were under the detection limit. In two samples we recovered what first seemed to be *Staphylococcus aureus* but after DNA Sequencing were proven different *Paenibacillus* and *Bacillus* species (Lupine Protein Isolate and Rice Protein Concentrate). Therefore no evidence for Coagulase positive *Staphylococcus aureus* were found in any sample.

Table 7: Bacillus cereus (MYP and PEMBA) in dry protein (CFU/g)



Samp.	MYP	PEMBA
PI	$3.6*10^4$	$1.6*10^3$
LC	<1000	<1000
LI	$1.5*10^5$	$3.0*10^3$
KI	$1.5*10^3$	$2.0*10^3$
WI	<1000	<1000
MC	<1000	<1000
SC	<1000	<1000
WG1	$2.2*10^3$	$2.0*10^3$
RC	$4.5*10^4$	$5.0*10^4$
WG2	$1.5*10^3$	$1.0*10^3$
EA	<1000	<1000

Yeast and molds

In all the samples, the concentration of yeast and molds were very low, with none being significally over the detection level of 1000 CFU/g (Tab. 8). In both Wheat gluten samples were yeast and molds detected, but the numbers were almost under the detection limit.

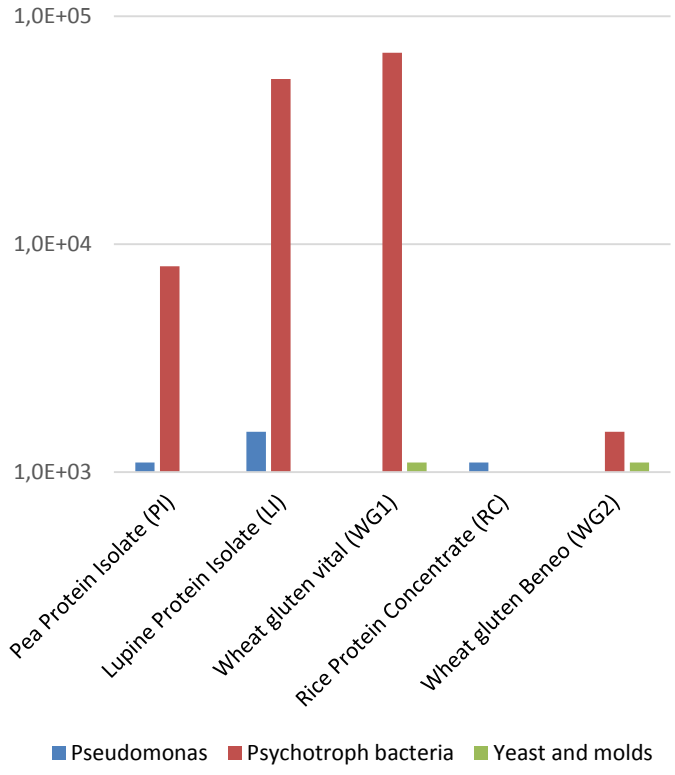
Pseudomonas aeruginosa

In all the samples, the concentration of *Pseudomonas aeruginosa* was under or slightly over the detection level of 1000 CFU/g (Tab. 8).

Psychotrophic microorganisms

In four of the eleven samples psychotrophic microorganism were found, with Pea protein Isolate and Wheat gluten Beneo with concentrations at slightly over the detection limit of 1000 CFU/g and Lupine protein Isolate and Wheat gluten from vital at levels between 10^4 and 10^5 CFU/g (Tab. 8).

Table 8: *Pseudomonas aeroginosa*, psychotrophic bacteria, yeast and moulds (YGC) in dry protein (CFU/g)

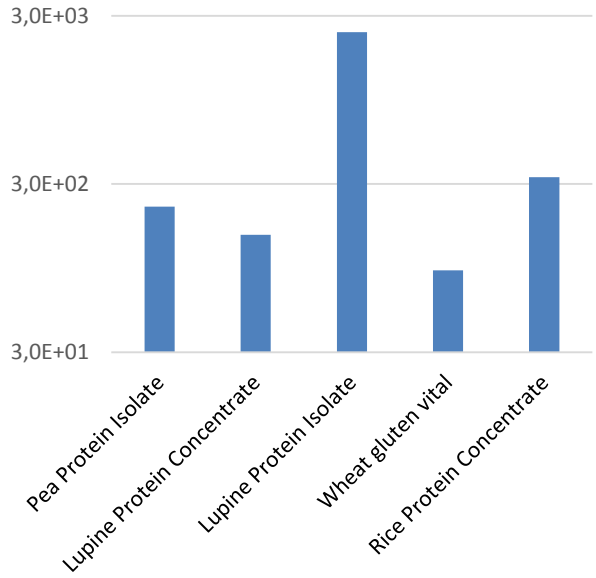


Samp.	*Pseudo-monas*	Psycho-troph b.
PI	$1.1*10^3$	$8.0*10^3$
LC	<1000	<1000
LI	$1.5*10^3$	$5.3*10^4$
KI	<1000	<1000
WI	<1000	<1000
MC	<1000	<1000
SC	<1000	<1000
WG1	$1.0*10^3$	$6.9*10^4$
RC	$1.1*10^3$	<1000
WG2	<1000	$1.5*10^3$
EA	<1000	<1000

Clostridium spp.

In 6 of the 11 samples the concentration of Clostridia was under the detection limit at 30 CFU/g. In the 5 other samples the concentration of *Clostridium* spp. was under the Guidance level of the Swiss Hygiene regulation of 2005, which lay at 10^4 CFU/g (Tab. 9). In three samples we detected *Clostridum perfringens* through plate cultivation, but this couldn´t be proved by DNA Sequencing. These samples were Lupine Concentrate and Isolate and furthermore Rice Protein Concentrate.

Table 9: Clostridium spp. and C. perfringens in dry protein (CFU/g)

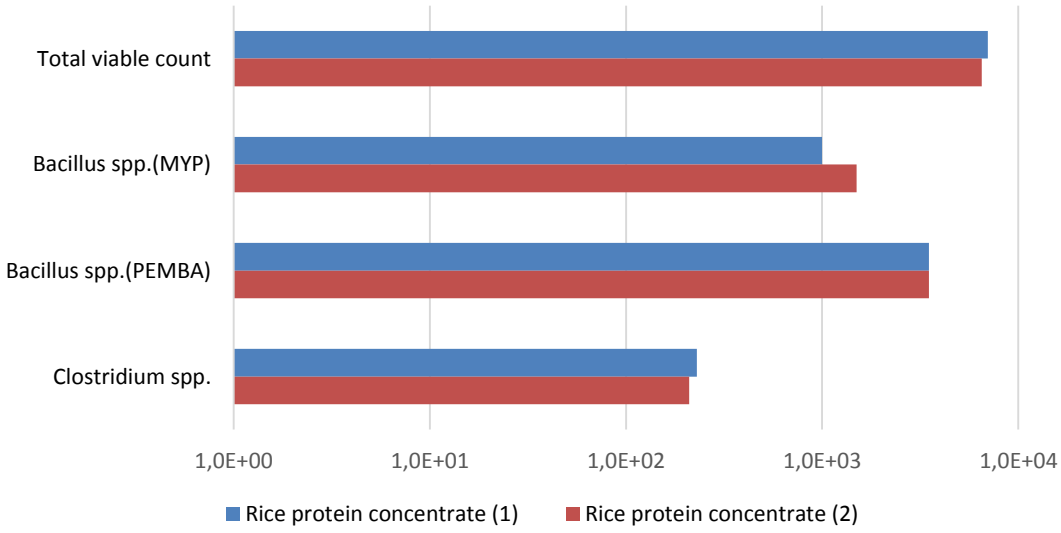


Samp.	*Clostridium* spp.
PI	$2.2*10^2$
LC	$1.5*10^2$
LI	$2.4*10^3$
KI	<30
WI	<30
MC	<30
SC	<30
WG1	$0.92*10^2$
RC	$3.3*10^2$
WG2	<30
EA	<30

4.2. Microbial examination of extruded protein

The extrusion step removed all viable microorganisms. In ten of the eleven samples there were no more microorganisms of any kind detected. The only sample were spore forming bacteria were detected was extruded Rice Protein Concentrate 100 % from Remypro N80. Two samples were taken, which showed almost the same concentrations of different bacteria, most of them spore-forming (Tab. 10). The Total viable count was reduced by almost three decimal power. *Bacillus* spp. were reduced by one decimal power. *Clostridium* spp. were recovered in almost the same concentration as in the raw protein powder.

Table 10: Bacterial growth in extruded Rice Protein Concentrate (CFU/g)



4.3. pH-Measuring of extruded product

The pH-value was measured in distilled water and in Ringer solution. In water the pH-values didn´t differ much, with Ringer solution, there are some samples that show a significant lower pH-value, which are potato protein + wheat gluten with a pH-Value of 4.6 and Rice protein concentrate with 5.7. The other nine samples are all between pH 6-7 (Table 11).

Table 11: pH of the extruded product in HQ water and Ringer Solution

Extrudate	HQ dest water	Ringer Solution
Lupine extract Lupidor 100%	7.20	6.7
Pea protein 50% +Egg yolk 50%	7.36	6.7
Milk protein 100%	7.13	6.3
Pea protein 100%	7.35	6.8
Wheat gluten Vital 100%	7.12	6.2
Pea protein 50%+ Whey protein 50%	7.15	6.5
Wheat gluten Beneo 100%	7.17	6.6
Soya Alpha 100%	7.33	6.7
Pea protein 30% + Lupi E 70%	7.24	6.5
Potato protein + Wheat gluten	6.78	4.6
Rice protein concentrate 100%	7.02	5.7

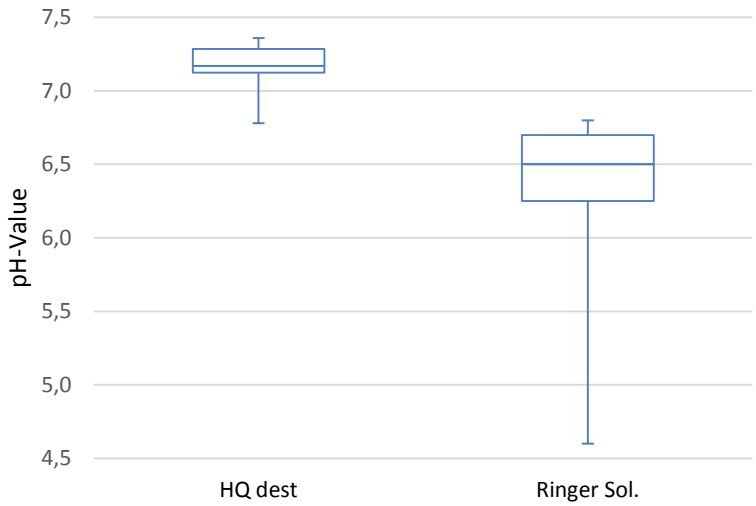


Figure 8: Box-plot of pH-Value in HQ dest and Ringer Solution

4.4. Agarose Gel electrophoresis

After the PCR we used an Agarose Gel electrophoresis to get DNA material for sequencing. We gave the names in Table 12 according to what we thought to be the microorganism. We used BAC for *Bacillus* spp., BC for *Bacillus cereus* and CP for *Clostridum perfringens*. The second letter and number combination were in row of examination of the bacterial culture. Figure 9 shows the Agarose Gel.

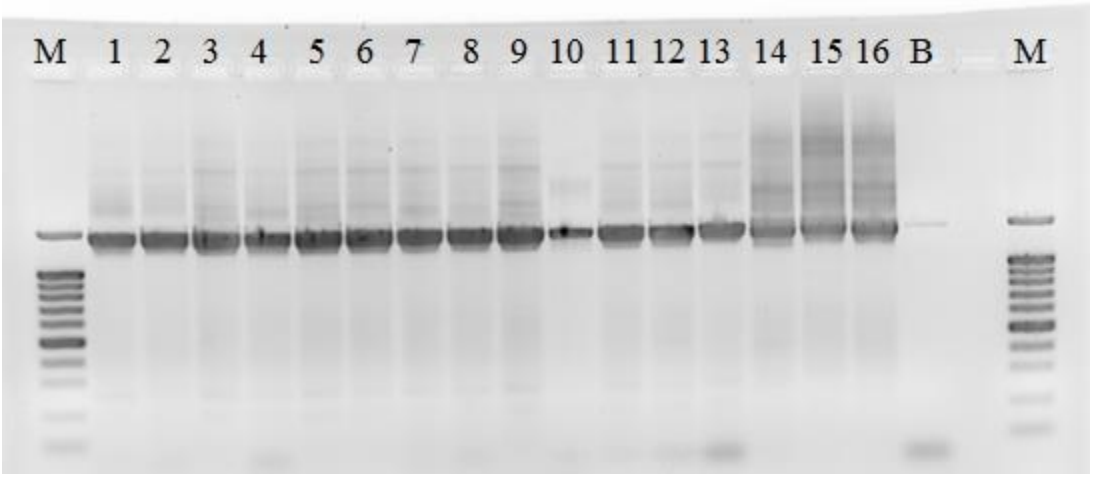


Figure 9: Agarose Gel Electrophoresis Gel

Table 12: Agarose gel electrophoresis: Cryoculture

1: BAC A 5.5 ng/µL	10: BC F3 8.5 ng/µL
2: BAC E2 4.1 ng/µL	11: BC C1 1.8 ng/µL
3: BC H3 2.2 ng/µL	12: BAC B 1.4 ng/µL
4: BAC I 1.6 ng/µL	13: CP C3 5.5 ng/µL
5: BC H2 2.1 ng/µL	14: CP C4 2.6 ng/µL
6: BC H1 4.3 ng/µL	15: CP C1 1.6 ng/µL
7: BC D 1.8 ng/µL	16: CP B1 1.6 ng/µL
8: BC C2 1.8 ng/µL	B: Blank
9: BAC E1 1.5 ng/µL	M: Marker

4.5. DNA Sequencing

These (Tab. 13) were the results we got back from the DNA-Sequencing. Unfortunately two of the Chello Sequencings showed no result, one of which we thought to be *B. cereus* (No.8) and *Clostridium perfringens* (No. 15). At chapter V. is an exact list of the DNA-Sequences we got back from the laboratory. Most of the *Bacillus* spp. we found were normal ground bacteria, which are not known to cause intoxication or infections. Just in two cases, we got a Cryoculture of *Bacillus cereus*, where one of these could be *B. cryotoxus* too.

Table 13: DNA-Sequencing of spore-forming bacteria in dry protein, results from the laboratory

1	*Bacillus licheniformis*	9	*Bacillus subtilis*
2	*Bacillus licheniformis*	10	*Bacillus amyloliquefaciens/subtilis*
3	*Bacillus licheniformis*	11	*Bacillus amyloliquefaciens/subtilis*
4	*Bacillus licheniformis*	12	*Bacillus cereus*
5	*Bacillus subtilis/amyloliquefaciens*	13	*B.subtilis/vallismortis/amyloliquef.*
6	*Bacillus subtilis/amyloliquefaciens*	14	*Paenibacillus sp.*
7	*Bacillus cereus/cryotoxus*	15	No result → Chello P0 failed
8	No result → Chello P0 failed	16	*Clostridium mesophilum*

In four samples we found different *Bacillus* species that are not known to be harmful to humans, these are *B. lichenformis, B. subtilis, B. amyloliquefaciens* and *Paenibacillus* spp. (Table 14). In Rice protein concentrate we could prove through DNA-Sequencing that *B. cereus*, which can be harmful to humans, was in the sample. The Rice protein concentrate had also a finding of *Clostridium mesophilum*, which is not known to cause harm in humans. We couldn´t prove the existence of *Clostridum perfringens*, which we found in the examination with culture media. The Cryocultures, which we thought be *Staphylococcus aureus*, were different *Bacillus* strains that had a similar growth on Baird Parker Agar.

Table 14: DNA-Sequencing results assigned to dry protein

	Bacillus lichenformis	*B. subtilis / amyloliquef.*	*Bacillus cereus*	Other spore-forming
Pea Protein Isolate	+			
Lupine Protein Isolate		+		
Potato Protein		+		
Wheat gluten vital		+		
Rice Protein Conc.			+	+

4.6. Detection of *Bacillus* spp. by different culture media

As we got different results for enumeration of *Bacillus* spp. on different culture media, we started a test row for enumeration of *Bacillus* spp. and *Bacillus cereus* on different media. For this cause, we plated different *Bacillus* strains (*B. licheniformis, B. subtilis* and *B. cereus*) on four different culture media (Fig. 10). Table 15 shows the results of the testing of the culture media.

Table 15: Test criteria for different Bacillus agar plates and results

Medium	Recognisability of *B. cereus*	Distinction of B. cereus to other *Bacillus* species	Growth of other *Bacillus* species	Growth of other bacteria	Possibility to quantify of *Bacillus* species
MYP	**++**	+	**++**	-	+
PEMBA	**++**	+	+	+/-	+/-
HCB	-	-	**++**	-	-
BBC	+	+	-	-	-

MYP (Mannitol-Egg Yolk- Polymyxin) from Oxoid

On this medium there is a good differentiation between *B. cereus* and other *Bacillus* strains. Next to a deep purple coloring of the media, *B. cereus* had dry-furrowed colonies in difference to other strains, which had a smooth and slimy growth. *B. licheniformis* and *B. subtilis* had an equally good growth and could be distinguished as *B. subtilis* grew in pink colonies and *B. licheniformis* grew in yellow colonies. These two *Bacillus* strains didn´t change the color of the medium to purple. Furthermore we didn´t discover any other growth than *Bacillus* spp. on this medium. Another advantage is that *Bacillus* spp. grow in small colonies, so counting of colonies is easier on this medium than on the others, which explains higher numbers of *Bacillus* spp. on MYP medium. These advantages make MYP the best choice for enumeration of *Bacillus* spp.

and *Bacillus cereus* from our point of view (Tab. 15). It is also the ISO 7932:2004 medium of choice for enumeration of *Bacillus* spp.

PEMBA (Polymyxin, Egg Yolk, Mannitol, Bromothymol Blue Agar) from Oxoid

On this medium there is a good differentiation between *B. cereus* and other *Bacillus* strains too. *B. cereus* is shown through a dark blue coloring of the medium; other *Bacillus* colonies color the medium bright green (Fig. 10). *B. subtilis* and *B. licheniformis* show a quite similar growth and can hardly be distinguished. A big disadvantage is that other bacteria can grow on this medium, so it is not very suitable for enumeration of *Bacillus* spp. in the raw material. Furthermore Bacillus spp. tend to grow in large colonies, which tend to overgrow each other, what makes enumeration difficult. This medium is a good choice for the verification of Bacillus cereus in comparison to other Bacillus strains after the enrichment on MYP.

HiChrome™ *Bacillus* from Fluka Analytical

The biggest disadvantage of this medium is that *Bacillus cereus* show hardly any until no growth on this medium (Fig. 10), which makes is unsuitable for the detection of *B. cereus* on food samples. Other *Bacillus* strains show good growth and easily distinguishable forms, which can be an advantage for further colonization and verification of other *Bacillus* strains. No other bacteria then bacillus can grow on this medium, which would make this medium a good choice for preparation of cryocultures with other *Bacillus* strains.

Brilliance™ *Bacillus cereus* from Oxoid

An advantage of this medium is that *Bacillus cereus* is the only *Bacillus* strain that can grow on this medium, showing through small blue points in the medium. No other *Bacillus* stains or any other microorganism can grow on this medium. A disadvantage is that no quantification is possible, just a yes or no answer to the question is there is B. cereus in the food sample. Another huge disadvantage is that no further enrichment of the culture is possible, which is not suitable for the preparation of cryocultures or DNA-sequencing.

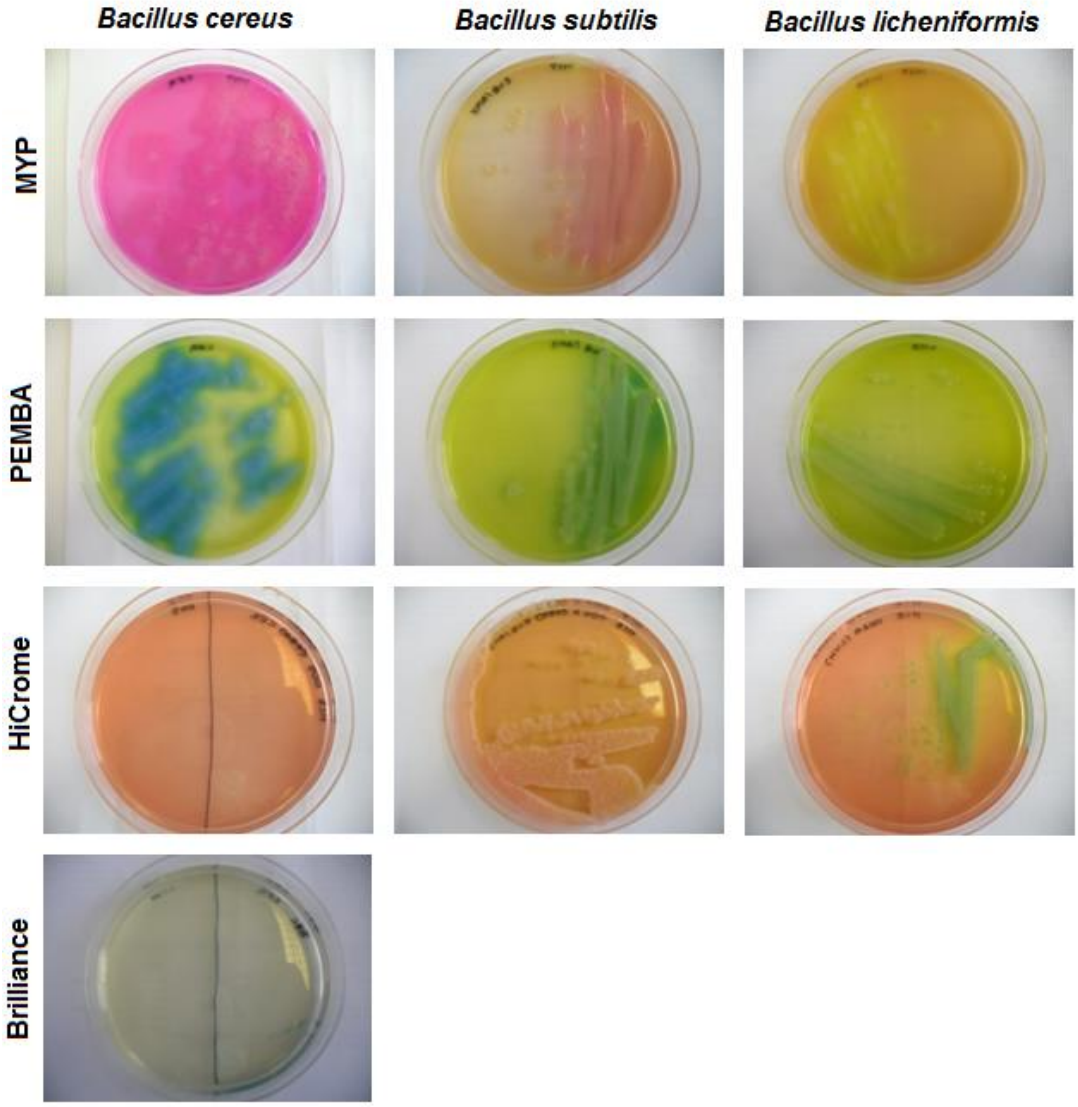


Figure 10: Agar plates with growth of different *Bacillus* species which were DNA-Sequenced to prove their identity.

4.7. Lipid body staining

We found the right dilutions and concentrations after several days of trying. Figure 11 shows the microscope picture. The lipid bodies are easily seen as dark purple to black spots in the B. cereus cells. Through the different colorization of Endospores, which can be seen as light green in the picture, it can be ruled out that the dark bodies are spores. Cytoplasm is colored red to a light pink and can be distinguished too. Some of the colors can´t be recognized that easily as they are through the microscope because of the photographic blur.

- Lipid bodies: dark purple to black
- Endospores: light green
- Cytoplasm: red to pink

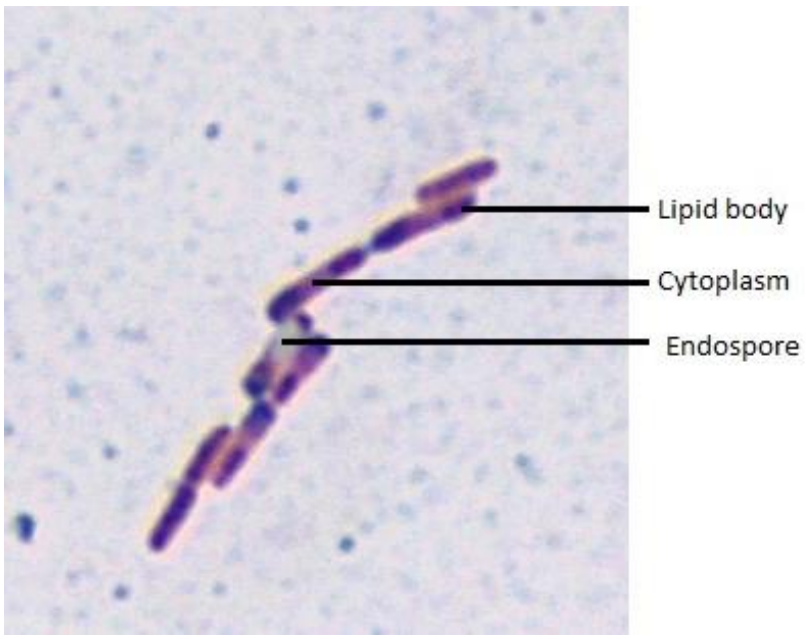


Figure 11: Photography of the lipid body staining done after the final recipe was found. It shows a *B. cereus* strain from Rice protein concentrate.

5. Discussion

5.1. Microbial examination of dry protein powder

For sample taking regarding to ISO 6887 a Stomacher bag with an integrated net inside was taken, because the dry plant protein produced a viscous slurry that could not be pipetted.

Most of the examined proteins showed hardly any contamination of bacteria and can be used for extrusion. These are: Potato Protein, Whey Protein Isolate, Milk Protein Concentrate, Soy Protein Concentrate and Hen Egg albumin (Tab.5). The concentration of bacteria was also compared to the guidance levels for Tofu from the DGHM (Tab.1).

Lupine Protein Concentrate, Pea Protein Isolate and the two different Wheat gluten samples showed no high contamination of any examined bacteria, but could contain harmful or food spoiling bacteria because of a high total viable count (Tab.5). These could be used for extrusion but there has to be a regular check on spore forming bacteria after the extrusion.

Two of the samples showed high concentrations of food spoiling and potentially harmful bacteria; they displayed critical values of spore forming bacteria (Tab. 6-9) and are therefore less suitable for usage in an extruded protein food than all the others. These two are Lupine Protein Isolate and Rice Protein concentrate.

In the raw protein samples many of the tested bacteria were present, except Listeria and Salmonella. There was a high amount of spore building ground bacteria (Tab. 7, 9) which is not unlikely considering that most of the plant protein grows near the ground (Mwangi, 2008). In the animal protein, no sporeforming bacteria were found. Furthermore the animal protein samples were less contaminated than the plant protein which can also be seen at Notermans et al. (1997).

In the plant protein were many psychotropic bacteria (Tab. 8), which are expected to grow under warmer conditions too, therefore the count for them can be seen as a fraction of the overall bacterial count at 37 °C (Scott et al,

1982). From the growth pattern of the psychotrophic bacteria, some could be identified to be *Bacillus* spp. which can grow at 4 °C according to Arntfield (2004). An anaerobic examination of psychotrophic bacteria wasn´t done, therefore there is no estimation of psychotrophic *Clostridium* spp. in the samples, but considering that most of the sporeforming bacteria can be found in soil and survive temperatures below freezing, the presence of spores of *Clostridium* spp. at 4 °C can be considered.

Low contamination of molds and yeast were in the samples, which is normally a problem with plant protein (Scudamore et al, 2004). Just some individual colonies could be found, which could be introduced at our laboratory.

Enterobacteria were found in acceptable amounts and could be an indicator of low hygiene during the packaging (Filho et al, 2005).

5.2. Microbial examination of extruded protein

The sample preparation could not be done regarding ISO 6887, because bacteria in the extruded sample would most likely be inside the extrudate and not on the surface of it, as it would be for the case for meat samples (Gill & Newton, 1977). Therefore the samples were homogenized with a decontaminated laboratory mixer. Then the sample was transferred sterile into the mixer and blended to a particle size of approximately 1-2 mm. After that exactly 10.0 g grained sample was aseptically transferred to a Stomacher bag and mixed with 100 mL buffered peptone water and mixed in a Stomacher Laboratory mixer as it is regarded in ISO 6887.

No viable bacteria survived the extrusion temperatures but spores could, so all but one of the examined samples were sterile. Just in one sample, a survival of spore formers was found, and that was the Rice Protein Concentrate. *Bacillus* strains and *Clostridium* strains could be found in the extruded sample in a still high count. This sample shouldn´t be used in the extrusion process, because *Bacillus* and *Clostridium* strains are shown to grow at 4 °C, a. k. the fridge and are normally inhibited by other aerobic or anaerobic bacteria, which

in this case are eliminated through extrusion, therefor the spores of *Bacillus* spp. or *Clostridium* spp. could grow without inhibition, making them a source for food poisoning and intoxication (Setlow, 2006; Setlow & Johnson, 2007). Therefore the Rice protein should be considered to be exchanged with another source of protein or another distributor of Rice protein should be tested for spore forming food spoiling bacteria (Akdogan et al, 1997).

5.3. pH-Measurement of extruded protein

Most of the extruded proteins have a pH value around 7 which provides ideal growth conditions for most bacteria, so the pH value cannot be used as a step in the hurdle system. Only Rice protein Concentrate and the mixture of Potato Protein and Wheat gluten made the pH value decrease a little bit, but hardly enough to protect the extruded food product from spoilage through bacteria (Wuytack & Michiels, 2000). An addition of different sauces or spices can be kept in mind, to make the product more acidic but that will be a challenge for the people that make the final recipe.

5.4. DNA Sequencing

The only sample where *Bacillus cereus* was found was the Rice Protein Concentrate. In all the others, where *Bacillus* species where found, it showed that they were *Bacillus lichenformis* or *Bacillus subtilis/amyloliquefaciens* which are normally not harmful to humans (Helgason et al, 2000; Radnedge et al, 2003). In addition, other spore forming bacteria where found in Rice Protein Concentrate, like *Paenibacillus* and *Clostridium mesophilum*. *Paenibacillus* can assimilate ammonia and therefore can cause a bad smell and a high pH value. *Clostridium* can assimilate different bad smelling and tasting gases. For *Clostridium mesophilum* also known as *C. amylolyticum* there is shown no harm for humans, but they can rot the product through fermentation of protein or starches, as this species is closely related to *Clostridium frigidicarnis* (Song & Dong, 2008), which is a known spoiling bacterium for red meat (Broda et al, 1999; Adam et al, 2011).

5.5. Detection of *Bacillus* spp. in different culture media

On MYP, developed by Mossel et al. (1967) *B. cereus* is easily distinguishable to other *Bacillus* strains, other Bacteria are repressed and a quantification of *B. cereus* is possible. This is also the media, which is in the ISO for *Bacillus* spp. detection.

On PEMBA, developed by Holbrook and Anderson (1980) other bacteria were not as far repressed as they are on MYP. *Bacillus* strains were distinguishable and *B. cereus* growth led to color change in the medium.

On HiCrome™ there was no detectable growth of *B. cereus* that were found in the plant protein samples. This medium could be taken for distinguishing between other *Bacillus* strains, because of the high spectrum of growth forms and colors.

Brilliance™ *Bacillus cereus* only showed *B. cereus*. Cloke et al. (2003) reports good growth of *Bacillus* spp. on this media. In our case, *Bacillus cereus* didn´t grow in colonies on this medium, this could be of a different strain of *Bacillus spp.*.

Němečková et al. (2011) did a quite similar research, but they had good growth of *Bacillus cereus* on the two chromogenic media. There is a possibility that they had other *B. cereus* strains, which grow better on this medium (Fig. 10).

5.6. Lipid body staining

As *Bacillus cereus* is one of the only *Bacillus* strains that develops Lipid bodies, Lipid body staining was a good way to see this characteristic feature of *B. cereus* in *Bacillus* cultures; still it took a while until the staining process was made perfect. With the first tries, the lipid bodies couldn´t be seen, so the process with Sudan black we got from Holbrook and Anderson (1980), developed by Schlegel et al. (1970) was changed. After some changes to concentrations of the different solutions and a change in staining times the lipid bodies were colored separately (Fig. 11). This method shows quite good results, but PCR and electrophoreses are much more accurate (Shamala et

al., 2003). Still it is a faster way to have an impression if the *Bacillus* strain is *Bacillus cereus*.

Other lipid body staining methods like Nile blue A (Ostle & Holt 1982) or Nile red (Gorenflo et al. 1999) were not tested, because they are more specific to Polyhydroxyalkanoates and won´t stain all lipid bodies that occur in *Bacillus* spp..

6. Conclusion and future perspectives

The first conclusion is that there is still much work to do in the microbiotical examination of extruded vegetable proteins. After the extrusion step, the product should be safe if there is no high contamination of *Bacillus* spp. or C*lostridium* spp. in the raw protein powder, which was a problem with the protein obtained from rice. Still there should be a microbial check for sporulating bacteria after the extrusion step for the finished product to rule out the intoxication with bacteria such as *B. cereus* or *Cl. perfringens*. If, after the extrusion, there is no check for other viable bacteria, there should be a very strict control of the handling of the product to prevent recontamination with pathogenic or food spoilage bacteria.

Future perspectives are the total elimination of endotoxic spores in the finished product, be it through the addition of antimicrobial ingredients, the pre-evaluation of raw material or the establishment of inhibitory microbiota in the finished product. As nobody has ever produced a similar product, there is no definite answer to which bacteria can actually grow and survive on the extruded product, just estimations can be given. This might not be that important to the product after extrusion as there are no viable bacteria left, but it will be of interest once the product is packed, spiced and flavored. There still has to be work done in the case of storage and stability for example, if the finished product is safe and stable at room temperature or if it must be kept at fridge temperatures or even frozen.

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8. List of 16S Sequences

***Bacillus licheniformis* from Lupine Isolate**

TACTGCAAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACG
TGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAA
CCGCATGGTTCAATCATAAAAGGTGGCTTTCAGCTACCACTTGCAGATGGACCCGCGGCGCATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGT
CTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAAC
AAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGG
TTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAG
TGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTG
GCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGC
AGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACG

***Bacillus licheniformis* from Rice Concentrate**

TACTGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGT
GGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAAC
CGCATGGTTCAATCATAAAAGGTGGCTTTCAGCTACCACTTGCAGATGGACCCGCGGCGCATTAGCTA
GTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTC
TGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACA
AGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGT
TTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGT
GCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGG
CGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCA
GCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGATTTGACGGCG

***Bacillus licheniformis* from Pea Isolate**

ATGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGG
GTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCG
CATGGTTCAATCATAAAAGGTGGCTTTCAGCTACCACTTGCAGATGGACCCGCGGCGCATTAGCTAGT
TGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTG
ACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAG
TACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTT
CTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGC
AGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCG
AAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGC
AAACGCATTAAGCACTCCGCCTGGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGATTTGACG

***Bacillus licheniformis* from Pea Isolate**

TACATGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACG
TGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAA
CCGCATGGTTCAATCATAAAAGGTGGCTTTCAGCTACCACTTGCAGATGGACCCGCGGCGCATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGT
CTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAAC
AAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGG
TTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAG
TGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTG

GCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCTCC

***Bacillus subtilis/amyloliquefaciens* from Pea Isolate**

ATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGG
GTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCG
CATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTT
GGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGA
CGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGT
GCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAG
CCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTC
TTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCA
GAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGA
AGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCT
AACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGCCC
GCACAAGCGGTGGA

***Bacilus subtilis/amyloliquefaciens* from Pea Isolate**

TACTGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGT
GGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAAC
CGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAG
TTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCT
GACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAA
GTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTT
TCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTG
CAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGC
GAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAG
CTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAACTCAAAGGAATTGAC

***Bacillus cereus/cytotoxicus* from Rice Concentrate**

TACTGCAAGTCGAGCGAACGGATTAAGAGCTTGCTCTTAAGAAGTTAGCGGCGGACGGGTGAGTAACA
CGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATCTAG
CACCGCATGGTGCAAGATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAG
TCTGACGGAGCAACGCCGCGTGAGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTTAGGGAAGAA
CAAGTGTGAGTTGAATAAGCTCATGCCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCG
GTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGA
GTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGT
GGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTG
AAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAG

***Bacillus subtilis* from Potato Isolate**

ACTGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTG
GGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTGAACC
GCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGT
TGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTG
ACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAG
TGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTT
CTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGC
AGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCG
AAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATAC

CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGC
TAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAGGA

***Bacillus amyloliquefaciens/subtilis* from Potato Isolate**

TACTGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGT
GGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGTTTGAAC
CGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAG
TTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCT
GACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAA
GTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTT
TCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTG
CAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGC
GAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAG
CTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGC
CCGCACAAGCGGTGGAGCA

***Bacillus amyloliquefaciens/subtilis* from Potato Isolate**

ACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGT
GGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGTTTGAAC
CGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAG
TTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCT
GACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAA
GTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTT
TCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTG
CAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGC
GAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAG
CTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGAC

***Bacillus cereus* from Rice Concentate**

TACATGCAGTCGAGCGAACGGATTAAGAGCTTGCTCTTAAGAAGTTAGCGGCGGACGGGTGAGTAACA
CGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATCTAG
CACCGCATGGTGCAAGATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAG
TCTGACGGAGCAACGCCGCGTGAGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTTAGGGAAGAA
CAAGTGTGAGTTGAATAAGCTCATGCCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCG
GTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGA
GTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGT
GGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTG
AAGTTAACGCATTAAGCACTCCGCCTGGGGGAGTACGGCCGCAAG

***Bacillus subtilis/vallismortis/amyloliquefaciens* from pea isolate**

TACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACG
TGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAA
CCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTA
GTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTC
TGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACA
AGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGT
TTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGT
GCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGG

CGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCA
GCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCACGCGAAG

***Paenibacillus* sp. From Rice concentrate**
CATGCAAGTCGAGCGGAGCGGATGGAGTGCTTGCACTCCTGATGCTTAGCGGCGGACGGGTGAGTAAT
ACGTAGGTAACCTGCCCTTAAGACCGGGATAACTCACGGAAACGTGGGCTAATACCGGATAGGCGATT
TCCTCGCATGAGGGAGTCGGGAAAGGCGGAGCAATCTGCCGCTTATGGATGGACCTACGGCGCATTAG
CTAGTTGGTGGGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACA
CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGCAA
GTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGA
ACGCTATGGAGAGTAACTGTTCCATAGGTGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCG
GTCATGTAAGTCTGGTGTTTAAACCCGGGGCTCAACTCCGGGTCGCATCGGAAACTGTGTGACTTGAG
TGCAGAAGAGGAAAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTG
GCGAAGGCGACTTTCTGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGCCGA
AGTTAACA

***Clostridium mesophilum* from rice concentrate**
CACATGCAGTCGAGCGAGGGAGTACCTTCGGGTACAACCTAGCGGCGGACGGGTGAGTAACACGTGGG
CAACCTGCCTTGTAGTGGGGGATAGCCCTCCGAAAGGAGGATTAATACCGCATAACATCATTTTATCG
CATGGTAGAATGATCAAAGGAGTAATCCGCTACAAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAG
GTAACGGCTCACCAAGGCCACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGC
AACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAAGCTCTGTCTTCAGGGACGATAATGACGGTAC
CTGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCC
GGATTTACTGGGCGTAAAGGGAGCGTAGGCGGATGTTTAAGTGGGATGTGAAATACCCGGGCTCAACT
TGGGTGCTGCATTCCAAACTGGATATCTAGAGTGCAGGAGAGGAAAGTGGAATTCCTAGTGTAGCGGT
GAAATGCGTAGAGATTAGGAAGAACACCAGTGGCGAAGGCGACTTTCTGGACTGTAACTGACGCTGAG
GCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTA
GGTGTGGGGGATTCGATTCCTCCGTGCCGCCGTTAACACAATAAGTATCCCGCCTGGGGAGTACGGTC
GCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTG

***Bacillus lichenformis* from pea isolate**
TACTGCAAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACG
TGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAA
CCGCATGGTTCAATCATAAAAGGTGGCTTTCAGCTACCACTTGCAGATGGACCCGCGGCGCATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGT
CTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAAC
AAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGG
TTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAG
TGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTG
GCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGC
AGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACG

***Bacillus licheniformis* from pea isolate**
TACTGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGT
GGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAAC
CGCATGGTTCAATCATAAAAGGTGGCTTTCAGCTACCACTTGCAGATGGACCCGCGGCGCATTAGCTA
GTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTC
TGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACA
AGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGT
TTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGT
GCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGG

CGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCA
GCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGATTTGACGGCG

***Bacillus lichenformis* from pea isolate**

ATGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGG
GTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCG
CATGGTTCAATCATAAAAGGTGGCTTTCAGCTACCACTTGCAGATGGACCCGCGGCGCATTAGCTAGT
TGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTG
ACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAG
TACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTT
CTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGC
AGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCG
AAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGC
AAACGCATTAAGCACTCCGCCTGGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGATTTGACG