HAMK Häme University of Applied Sciences

Development Of High Efficiency Low Tech Inoculation and Growing Techniques for Organic Reishi

Bachelor's thesis

Climate Smart Agriculture

Spring 2024

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Climate Smart Agriculture Year 2024
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Subject Development of High Efficiency Low Tech Inoculation and Growing Technics for Organic Reishi
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This thesis examines the optimization of low-tech inoculation and growing techniques for the cultivation of organic Ganoderma Lucidum (Reishi) mushrooms in laboratory settings. Accredited by Kääpä Biotech, the research aims to find the most useful methodologies and conditions for cultivating Reishi, harnessing accessible and sustainable low-tech processes.

The study consists of two seperate experiments designed to observe various parameters influencing the growth of Reishi, including environmental factors , inoculation methods, and substrate composition. The results from these experiments provide valuable insights into the development of a high-efficiency protocol for cultivation of Reishi mushrooms with minimal technological involvements.

Other than Reishi, the literature review encompasses a comprehensive analysis of various medicinal mushrooms, including Lion's Mane (Hericium erinaceus), Shiitake (Lentinula edodes), Maitake (Grifola frondosa), Cordyceps (Cordyceps sinensis), Turkey tail (Trametes versicolor), and Chaga (Inonotus obliquus), respectively. This broader exploration sets the context for understanding the unique requirements and features of Reishi, placing it within the broader framework of medicinal mushroom cultivation and the realm of nutraceuticals.

Offering insights into scalable and sustainable methods for organic Reishi production, the findings of this thesis hold practical significance for Kääpä Biotech. The application of low-tech techniques not only contributes to the economic viability of mushroom cultivation but also aligns with eco-friendly practices but also. The results can conceivably inform the development of commercially viable cultivation processes for Reishi and other medicinal mushrooms, hence advancing the goals of Kääpä Biotech in the field of mycological research and product development.

Keywords Reishi, Ganoderma Lucidum, Fungi, Mushroom, Organic, Kääpä Biotech
Pages 35 pages and appendices 2 pages

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1 Introduction

The primary reference to European utilization of mushrooms as medication comes from Ötzi, the "Iceman", around 3500 BCE (Gründemann et al., 2020). Amongst several items that were found on his body, were a handful of fungal objects: two fruitbody pieces of the polypore *Fomitopsis Betulina* (formerly known as *Piptoporus betulinus*) and a large quantity tinder material prepared from *Fomes Fomentarius* (Peintner et al., 1998). As claimed by Pleszczyńska et al. (2017), the fungus has a long tradition of being applied in folk medicine as an antimicrobial, anticancer, and anti-inflammatory agent, and it is probably due to the curative properties, pieces of its fruiting body were carried by Ötzi the Iceman.

Figure 1. Birch Polypore, Piptoporus betulinus, found amongst Ötzi's belongings (Mossy Creek Mushrooms website, 2019)

While pharmaceuticals derived from plants play a crucial role in an empirical-based medicine in the Western Hemisphere, medicinal mushrooms are primarily used as dietary supplements without officially stating their medical purpose. Research and real-life examples from Asian medicine suggest that fungi have great potential for pharmacological applications as well. (Gründemann et al., 2020).

A diverse group of eukaryotic organisms, edible and medicinal fungi come in various forms, including *Coriolus versicolor* (Turkey Tail), *Ganoderma lucidum* (Reishi), *Cordyceps sinensis*, *Pleurotus ostreatus* (Oyster), and *Grifola frondosa* (Maitake). They've shown a wide range of pharmaceutical properties, like antiviral, anti-inflammatory, and neuroprotective effects. For thousands of years, these fungi have been consumed both as food, providing essential nutrients, and as traditional medicines in China, known for enhancing blood circulation, strengthening vitality, cooling, and resolving congestion. (Xu et al., 2022)

Cancer, a major global cause of death, resulted in nearly 10 million fatalities in 2020. Thus, there's an urgent need to explore strategies for cancer prevention and treatment. Numerous studies have reported that the primary bioactive compounds in these fungi, mainly polysaccharides and triterpenoids, exhibit various anti-cancer effects through multiple mechanisms. This includes slowing cell growth, preventing metastasis, promoting apoptosis and autophagy, overcoming drug resistance, and modulating immune responses. These findings suggest a significant potential for edible and medicinal fungi in the fight against cancer. (Xu et al., 2022)

Due to the scarcity of many medicinal mushrooms in their natural habitat, the supply of fungal fruiting bodies falls short in both the food market and pharmaceutical product manufacturing. Consequently, there has been a substantial push towards cultivating these fruiting bodies artificially over the past four decades. Diverse cultivation methods have emerged, encompassing traditional practices like growing on wood logs and beds, as well as newer approaches using substrates like bags, bottles, and more. (Nikšić et al., n.d.)

One major advantage of this artificial cultivation is its cost-effectiveness, even though it does require a significant amount of time to achieve large-scale production. This process primarily utilizes agricultural, wood, and food industry waste as substrates. These materials are treated to remove lignin and are enriched with proteins and valuable pharmaceutical compounds. (Nikšić et al., n.d.)

The wholesale product line is seeing a significant surge in demand. Consequently, Kääpä Biotech is moving into the Series A investment stage to expand production capabilities to meet the rising global need for these medicinal mushroom extracts. (Kääpä Biotech, 2021)

The medicinal mushroom market has undergone rapid growth in recent years. Thus far, it has lacked options that are of high quality, innovative, transparent, and sustainable. The increasing desire for top-notch, organic medicinal mushroom ingredients have motivated Kääpä Biotech to create the Nordic Mushrooms product line. This product line encompasses organic Chaga *(Inonotus obliquus*), Reishi (*Ganoderma lucidum*), Shiitake (*Lentinula edodes*), Lion's mane (*Hericium erinaceus*), and Maitake (*Grifola frondosa*) grown in Finland. (Kääpä Biotech, 2021)

The writer decided to include some of the prominent mushroom types under Kääpä Biotech inventory in the following literature review. Although the thesis topic and research are about Reishi (hence its location in the hierarchical order in this report), it is important to note that all the other mushroom types play a significant role for commissioner and should be acknowledged, even solely for juxtaposed research purposes.

2 Reishi *(Ganoderma Lucidum)*

Reishi, often hailed as the king of herbal medicines, holds a position of high esteem among herbalists, even surpassing ginseng. While some individuals opt to brew *Reishi* teas, it is predominantly consumed for its medicinal properties due to its intensely bitter and woody flavour. Scientifically known as *Ganoderma lucidum*, its name derives from the Latin words "gan" meaning "shining", "derma" meaning "skin," and Latin for "lucidus" meaning "brilliant." *Reishi* is also referred to as the "Ten-thousand-year mushroom" and the "Mushroom of immortality." Its most notable characteristic is its glossy lacquered appearance, featuring a kidney-shaped cap that may exhibit spores resembling sandpaper. *Reishi* mushrooms come in a variety of colours, including red, white, black, blue, yellow, and purple. This mushroom thrives in dense and humid coastal regions of China, often found on decaying stumps of chestnut, oak, and other broad-leaf trees. (Halpern, 2007, pp. 55–56)

Figure 2: *Ganoderma Lucidum* healthy fruiting body, prior to appearance of mold during preliminary experiment (Kääpä Biotech, June 2023)

2.1 Historical Use

With a rich heritage in ancient Chinese remedies, *Ganoderma Lucidum* is a mushroom variety that is thought to have longevity-enhancing properties and contribute to overall well-being (Unlu et al., 2016).

According to the ancient Chinese manuscript Shen *Nong Ben Jing*, dating back to around 500 CE, *Ganoderma lucidum* is described as having beneficial effects on vitality, cognitive function, and memory preservation. It is believed to rejuvenate the body and mind, slow down the aging process, and promote mental stability. The significance of maintaining memory in old age can be attributed to Taoist principles, which suggest that illnesses are a result of past wrongdoings. In order to heal, one must recall and document these transgressions, subsequently destroying the records. This philosophy places great importance on memory for the sake of maintaining good health and longevity. (Halpern, 2007, p. 57)

2.2 Cultural References

Reishi mushroom has an intriguing history. A poem by the philosopher Wang Chung from the first century mentions the Taoist priests' utilization of mushrooms in their pursuit of elevated consciousness:

"They dose themselves with the germ of gold and jade.

And eat the finest fruit of the purple polypore fungus.

By eating what is germinal, their bodies are lightened.

And they are capable of spiritual transcendence."

(Halpern, 2007, p. 58)

Legend has it that in the first century, Taoist priests were the pioneers in exploring the properties of reishi. They purportedly incorporated the mushroom into magical potions believed to bestow longevity, eternal youth, and even immortality. These priests, practitioners of alchemy, were known for their enchantments and concoctions, often regarded as magicians or wizards in modern terms. It is important to note that alchemy marked the beginnings of chemistry, and the early healers, referred to as shamans, relied on nature's forces to treat the sick, making them the earliest doctors.

Lingzhi, a medicinal mushroom, has a longstanding history spanning over 2000 years, with its remarkable effects documented in ancient texts (Wasser & Weis, 1999). The artistic depiction of *Ganoderma lucidum* began around 1400 AD and became associated with

Taoism. Interestingly, G. lucidum images were not limited to religious contexts but also found their way into paintings, carvings, furniture, and even women's accessories. A significant milestone in herbal literature was the publication of the Shen Nong Ben Cao Jing during the Eastern Han dynasty of China (25-220 AD), which served as the first comprehensive book solely dedicated to describing herbs and their medicinal properties. (Wachtel-Galor et al., 2011)

2.3 Cultivation Methods

Ganoderma lucidum is a type of Basidiomycete fungus that breaks down wood, primarily relies on lignin. Lignin is a complex compound found in the cell walls of plants, which provides structural support and strength to plant tissues, allowing them to stand upright and resist mechanical stress. In that sense, the lignin is used as a source of carbon. (Liu et al., 2012) Due to its rarity and slow growth in natural settings, techniques and culture media have been developed and refined to enable large-scale production of *Ganoderma Lucidum* under controlled environments. (Zhou, 2017) An enhanced cultivation method called "short wood-log" has been developed and increasingly utilized in industrial production. GL cultivation has gained popularity in numerous regions of China. (Tong et al., 2020)

Substrates options are diverse in industrial environments, as Kääpä Biotech use sawdust and spelt, but the options are quite varied, as several studies demonstrate, such as Ozcariz-Fermoselle et al (2018), where pecan woodchips, shell, and pericarp (outermost layer of a fruit) can be utilized in cultivation of Reishi.

2.4 Medicinal Effect

Ganoderma lucidum has been utilized for many years in Asian nations for its medicinal properties and ability to enhance overall well-being and lifespan. Research conducted on G. lucidum has demonstrated its positive impact as a complementary therapy for cancer patients, without any apparent harmful side effects. (Sohretoglu & Huang, 2018)

Seweryn et al. (2021) demonstrated in detail *Ganoderma lucidum* polysaccharides medicinal effect when consumed as nutraceutical: “The pharmacologically active fraction of polysaccharides has antioxidant, immunomodulatory, and neurodegenerative and antidiabetic activities”.

Various research has demonstrated that GLP (*Ganoderma lucidum* polysaccharides) can control the production of various inflammatory cytokines in different models of inflammation and diseases, contributing to the immune response against infections (Chen et al., 2023).

1.4.1 Immune system support

Laboratory research and a handful of preclinical trials have suggested that G. lucidum carries promising immunomodulatory properties (Jin et al., 2016). The medicinal properties of these functions have been classified, including their ability to stimulate the body's natural defences, inhibit tumour growth, and regulate cell growth. Previous reports have highlighted the effectiveness of specific fractions of *Ganoderma lucidum* polysaccharides (GLP) in modulating the immune system. (Cai et al., 2017)

Both of the abovementioned sources mentioned that despite the existence of several researches in the topic, there is still not enough data to establish a firm scientific claim, although it is mostly a factor of time and amount of research being done in the future.

1.4.2 Anti-inflammatory properties

Acute inflammation arises from a complex cellular signalling process that safeguards and facilitates the healing of our body, contributing to overall well-being and good health. In contrast, chronic inflammation is strongly linked to the emergence of numerous autoimmune conditions such as rheumatoid arthritis, systemic lupus, polymyalgia, rheumatic ailments, as well as diseases like asthma, inflammatory bowel diseases, cardiovascular disorders, ulcerative colitis, Crohn's disease, and even certain types of cancer. An effective anti-inflammatory medication should possess the ability to selectively impede the progression of chronic inflammation while preserving normal physiological balance. Several natural remedies have been previously identified for their potential in targeting inflammatory cytokines. Among these*, Ganoderma lucidum* stands out as a notable example. (Bhardwaj et al., 2014)

1.4.3 Cardiovascular Health

Cardiovascular diseases (CVDs) relate to various heart and blood vessel disorders, including coronary heart diseases, cerebrovascular diseases, rheumatic heart diseases, and other related conditions. CVDs rank among the leading causes of illness and death worldwide, claiming the lives of approximately 17.9 million individuals annually. Numerous investigations have explored the impact of *Ganoderma lucidum* on certain metabolic indicators such as low-density lipoprotein (LDL), high-density lipoprotein (HDL), total cholesterol (TC), blood pressure, and oxidative damage. G. lucidum shows potential in reducing the risk of developing cardiovascular diseases. (Meng & Yang, 2019)

Numerous herbal medicines are believed to have potential in the treatment and control of cardiometabolic disorders and associated risk factors. *Ganoderma Lucidum* has gained significant interest for diverse applications, including certain aspects related to the prevention and treatment of cardiovascular and metabolic disorders, by improving key risk factors associated with cardiovascular health. Several laboratory-based experiments and animal studies have demonstrated that G. lucidum showcases properties such as antioxidant, antihypertensive, hypoglycaemic, lipid-lowering, and anti-inflammatory effects. However, when it comes to clinical trials, the observed health advantages have not been consistently reported. Among the potential benefits, the most convincing evidence to date lies in its ability to reduce blood sugar levels in individuals with type 2 diabetes or hyperglycaemia. (Chan et al., 2021)

3 Other Mushrooms

4 Lion’s Mane *(Hericulum Erinaceus)*

Also known by its scientific name *Hericium erinaceus*, Lion’s mane mushroom is endemic to the northern hemispheres of Europe and America but can be found in East Asia as well. Its unusual and exotic appearance granted its irregular names: Old Man’s Beard, Bearded Hedgehog, Monkey’s Mushroom and more. The mushroom prefers dying or dead broadleaf trees such as beech, walnut, and oak. In the wild, its dimensions are 5-20 cm across. Its tendrils hang from its rubbery base. Amongst its active ingredients are: hericenons A, B, C, D, E, F, G, and H, fatty acids (Y-A-2) while the mycelium contains diterpenes called erinacines that mimic the factor of nerve growth. One of the erinacines is an opioid, hence used as pain mitigator. (Halpern, 2007, p. 107-108)

Figure 3. Fully matured Lion’s Mane during growing season in Kääpä Biotech outdoor tents,

4.1 Historical use

In traditional Chinese medicine, Lion’s mane is prescribed for gastrointestinal illness, stomach disorders and ulcers. Native Americans in North America use Lion’s Mane as styptic, applied in its dried form to scratches and cuts to pause bleeding, and it was usually found in Native American’s medicine bag. A powder extract from the mushroom called “Houtu” is sold in China. Beyond its medicinal use, Lion’s Mane is also a culinary mushroom. Its flavor may give a hint of seafood, lobster or crab flavor, and the fact that its rubbery texture can remind of a squid might amplify the resemblance. Once quite a rare find in nature, Lion’s Mane can nowadays be found in different culinary establishments around the world. (Halpern, 2007, p. 107)

4.2 Cultivation Methods

Up to several years ago, Chinese pharmacies carried powders and pills made of *Hericulum erinaceus*. It is probable that very often their clients had no idea that its cultivating methods were developed in Sonoma County, California. This happened when a fellow mycologist informed Malcolm Clark that he had seen an unusual fruiting of *Hericulum erinaceus* on a tree in Glen Ellen, a small town in Sonoma County, about 80 km north of San Francisco. (Halpern, 2007, p. 109) Lab grown methods are also possible and quite successful. Substrates based on sunflower seeds, with or without the addition of wheat bran show high mycelial growth rate, and the addition of barley straw, or poplar sawdust was also tested but showed no statistical difference in growth rate. When supplemented with 20 ppm Manganese and 200 ppm ammonium ion, sunflower seed hull control demonstrated a tendency to higher yield. (Figlas et al., 2007)

4.3 Medicinal Effect

Despite its usage in China and Japan for many centuries, Lion's Mane was brought into science attention at the beginning of the 21st century. In his article in the International Journal of medicinal Mushrooms, (Halpern, 2007, p. 110) Dr. Takashi Mizuno of Shizouka University in Japan, pointed out the following:

- Studies (amongst other findings) also show how phenols and fatty acids from Lion's Mane mushroom may have chemotherapeutic effects on cancer, and how these molecules employ directly against cancerous cells.

- Polysaccharides produced by the fruiting body may assist against skin, oesophageal, and stomach cancer. These are also aiding the immune system of people with cancer in managing and controlling the aftereffect of chemotherapy.

Lion's Mane and Alzheimer

Dr. Mizuno's article was implying about possible treatment of Alzheimer disease using Lion's Mane. Being the most common form of an irreversible dementia, its symptoms include inability to speak or reason, memory loss, confusion, and disorientation. Scientists claim that it caused by plaque build-up around nerve cells in the brain and by distorted nerve fibres. It has no cure, and it is always fatal. Dr. Mizuno reported that compounds in Lion’s Mane (hericenones C, D, E, F, G, and H) may encourage production of protein called NGF (Nerve Growth Factor) required in developing and maintaining important sensory neurons in the brain. In other words, Lion’s Mane might have the ability to improve symptoms of Alzheimer. (Halpern, 2007, p. 110)

Lion’s Mane and Diabetes

A study (Cai et al., 2020) has shown that Lion’s Mane extracts regulated levels of blood lipids (fats), reduced blood glucose in diabetic rats, have antioxidant abilities and demonstrated significant shift in cholesterol levels when being fed with the mushroom concentrate daily (In a ratio of 1g/1kg body weight).

5 Shiitake *(Lentinus Edodes)*

Lentinus edodes, or as commonly known by its Japanese name, Shiitake, derives from the mushroom associated with the *shii* tree, and the Japanese word for mushroom – *take*. Thanks to Japan being an international leader in producing this type of mushroom, it is now widely known by this name (Halpern, 2007, p. 47-48).

The mushroom is recognized as a medicine and food in East Asian countries (Korea, Japan, China) for thousands of years. The pileus is dark brown at first and grows lighter as it biologically ages. The edges of the gills are serrated, while the spores are white. During spring or winter and in the wild, shiitake grows on dying or dead hardwood trees, such as mulberry, beech, etc (Halpern, 2007, p. 47-48).

Shiitake opts for shaded forest and the proximity of cold water. (Halpern, 2007, p. 47-48)

Figure 4. Shiitake Mushrooms (Nordic Mushroom website)

5.1 Historical Use

Shiitake is supposed to have been used in the ancient Japanese royal court as an aphrodisiac (Halpern, 2007, p. 49-50).

The log method of cultivating mushrooms was born due to Shiitake. About a 1000 years ago, a woodcutter named Wu San-Kwung of the Zheijang province, wanted to test his axe. He swung it against a fallen log on which Shiitake mushrooms grew. Days later, he noticed shiitake mushrooms growing where his axe struck the log. As an experiment, he cut the log in several different places, and once again, shiitake mushrooms fruited where his axe landed. In this way, the log method of cultivating mushrooms was born. As the story proceeded, mushrooms failed to grow on the log, much likely to Wu's despair. He attacked the log, beating it violently with the blade of his axe. When he returned to the scene, he discovered the "soak and strike" method of mushroom cultivation, in which logs are pound in a way that spores have more opening for germination. This method is still used in different places. (Halpern, 2007, p. 49)

5.2 Cultivation Methods

In nature, the shiitake fungus reproduces through spores released by mature mushrooms. Yet, when it comes to cultivation, relying on spore germination proves to be an unreliable method. Instead, the process involves introducing actively growing fungus to logs. Prior to this, the fungus is accustomed to the wooden environment by cultivating it on small wood pieces. These dynamic fungal cultures, known as spawn, serve as the vital material for mushroom cultivation. It is crucial for growers to utilize high-quality shiitake spawn of desirable variety, cultivated in a controlled environment devoid of unwanted fungi and bacteria, as the quality of the resulting crop is directly influenced by the quality of the spawn used. (Halpern, 2007, p. 49)

5.3 Medicinal Effect

Shiitake mushrooms have been used for a long time for their medicinal properties, which can help fight against cancer, reduce inflammation, protect against harmful substances, fight against fungal and bacterial infections, and support heart health. (Rahman & Choudhury, 2013)

In traditional Chinese medicine, shiitake is used to treat high cholesterol, atherosclerosis, colds, and flu (Halpern, 2007, p. 50).

Shiitake and Tooth Decay

Dental plaque is a film of bacteria that forms on teeth and can cause decay. Regular brushing is essential to prevent plaque. Researchers conducted tests using shiitake powder on plaque-causing bacteria, seeing a reduction in plaque formation. Rats fed shiitake extract showed fewer cavities compared to those without it. Shiitake demonstrated effectiveness against common mouth bacteria but not against non-oral microbes like Candida. These findings suggest shiitake could be valuable for dental health. (Halpern, 2007, p. 52-53)

Shiitake and Hepatitis

LEM (*Lentinula Edodes* Mycelia), extracted from shiitake mushrooms, shows promise against hepatitis B by stimulating antibody production. It, along with LAP (Latency Associated Peptide), exhibits antitumor properties. These extracts contain glucose, galactose, xylose, arabinose, mannose, fructose, nucleic acid derivatives, vitamin B compounds, and ergosterol. LEM and LAP activates the immune system, and in Japan, Lentinan (Shiitake mushrooms substance) is a medicine while LEM and LAP are food supplements. (Coates et al., 2010, p. 657)

Shiitake and HIV/Aids

In the early 1980s, physicians began experimenting with Lentinan to strengthen the immune system against HIV, the virus causing AIDS. Lentinan showed promise in increasing helper T cells, crucial for immune response. LEM, found in shiitake mushrooms, contains lignin that block HIV proliferation and protect T-helper lymphocytes. A protein called Lentin, isolated from *Lentinula edodes*, displayed antifungal and inhibitory effects on HIV-1 reverse transcriptase (used for DNA replication, repair, and mutagenesis) and leukemia cell proliferation. Studies conducted on AIDS patients using Lentinan showed increased lymphocytes, but sample size limitations prevented definitive conclusions. Lentinan, when combined with AZT or didanosine, exhibited potent anti-HIV activity, suppressing viral replication, and increasing lymphocyte counts. (Halpern, 2007, p. 51)

6 Maitake *(Grifola Frondosa)*

In English, Maitake is known as “Hen of the woods”. In Japanese, it means “dancing Mushroom (*mai* means “dance”; *take* means “mushroom). Its name origin depends on several narratives, in one of which the mushroom got its name because people danced in joy upon finding maitake in the wild. The story goes that in Japan’s feudal era, local lords paid tribute to shogun (military ruler) by presenting him with maitake mushrooms (amongst other gifts). The local lords are supposed to have offered anyone who found one the mushroom’s weight in silver, perhaps the cause of dance. Another narrative suggests the dancing mushroom got its name because of the coinciding fruit-bodies, that give the appearance of a kaleidoscope of butterflies. (Halpern, 2007, pp. 35–37)

Because of its size, Maitake is sometimes called the “king of mushrooms”. The mushroom’s scientific name is *Grifola frondosa*. *Grifola* is the name of a fungus found in Italy, and some scholars believe the fungus got its name from the griffin, the mythological beast with wings and head of an eagle and hind legs and tail of a lion. *Frondosa* means “leaflike”, as the overlapping caps of maitake growing in the wild will give the appearance of leaves. (Halpern, 2007, pp. 35–37)

One of maitake’s main features is that it grows in clusters. The caps, which are typically 10-12 centimeters across, overlap to form a sort of clump. A typical maitake cluster is about the size of a volleyball. Meanwhile, the stems are fused together. Maitake grows at the base of beech tree, oak, and other dying or dead hard woods. (Halpern, 2007, pp. 35–37)

A cluster can weigh about 36 kilograms and be about 50 centimeters in diameter. Maitake prefers temperate northern forests, and it is indigenous to Europe, Asia, northeast Japan, and the eastern side of the North American continent. (Halpern, 2007, pp. 35–37)

Figure 5. Maitake Mushrooms (mushroom council website)

6.1 Cultivation Methods

Maitake mushroom cultivation in Japan has a relatively short history of 15 years. Mass production started 10 years ago after studying its biology. Three cultivation methods were developed:

- Bottle culture, suitable for year-round mechanized cultivation with small bottle sizes resulting in smaller mushrooms.
- Bag culture, widely used in Japan, involving mixing sawdust with rice and wheat brans, packing the mixture in polypropylene bags, and forming square-shaped culture beds.
- Outdoor bed culture, an attempt under natural conditions, taking about 6 months from inoculation to fruiting body formation. This method harnesses the climate for growth. (Mayuzumi & Mizuno, 1997)

6.2 Medicinal Effect

On top of its culinary usage, the Japanese value Maitake for its medicinal properties. Traditionally, it was used in Japan as a tonic to increase vitality and boost the immune system, and it was believed to prevent high blood pressure and even cancer. That was what caused researchers to turn their attention to maitake's influence on these diseases about 50 years ago. Recently, maitake has become a popular subject of study. Macrophages play important roles in many different aspects of an organism's biology. They are involved in development, maintaining balance in the body (homeostasis), repairing damaged tissues, and defending against harmful pathogens. Resident macrophages act as guardians, constantly monitoring the body's tissues for any changes in their environment or threats from the outside. They play a crucial role in maintaining the stability and well-being of the body by responding to these changes and challenges in order to keep everything functioning properly. (Wynn et al., 2013)

In 1984, a Japanese scientist named Hiroaki Nanba discovered a substance in Maitake mushrooms that can activate macrophages. These substances, known as D-fraction, are made up of specific compounds called beta-glucan polysaccharides, particularly beta-D-glucan. Both the mycelia and the fruit body of the Maitake mushroom contain this D-fraction, which has the ability to stimulate the immune system. (Halpern, 2007, p. 37)

Maitake and Diabetes

Several studies suggest a strong correlation between compounds found in Maitake and its ability to show promising results in treating diabetes amongst mice and rats.

KK mice are a specific type of mice that are a bit overweight and have high levels of a hormone called Leptin. These mice have trouble processing glucose, which leads to a condition called impaired glucose tolerance. They also have a problem with insulin, a hormone that helps regulate blood sugar levels. In KK mice, insulin doesn't work properly, causing their bodies to produce too much insulin, a condition known as hyperinsulinemia. (Clee and Attie, 2007)

When a daily dose of 1g of powdered Maitake was administered orally to a KK mouse, it resulted in a decrease in blood glucose levels compared to the control group, whose blood glucose increased as they aged. Additionally, the levels of insulin and triglyceride in the mouse's blood showed a similar pattern to the changes in blood glucose when Maitake was consumed. (Kubo et al., 1994)

Maitake and Cholesterol

Cholesterol is a substance made by the liver that is important for the body's functioning. It helps with things like making new cells and producing hormones. There are two types of cholesterol: HDL (or "good") cholesterol and LDL (or "bad") cholesterol. HDL carries fat through the blood and prevents it from building up. LDL, on the other hand, can deposit fat in the liver and blood vessel walls, which can cause problems. Eating a lot of saturated fats can raise cholesterol levels in the blood. Having high cholesterol can lead to health issues like atherosclerosis and other problems. (Halpern, 2007, p. 39)

Other than regulating genes in skeletal muscles and improve glucose intolerance, Aoki et al. (2018) found that lipid soluble extracts derived from *Grifola frondosa* can, when administrated to high-fat diet-induced in obese mice, lowered the total blood cholesterol levels as well.

Harnessing Synergy: Amplifying the effects of combined substances

Synergy occurs when substances taken together amplify each other's effects. This is commonly observed with natural products and medications, grapefruit juice for example. Grapefruit juice blocks certain enzymes in the intestines, leading to reduced metabolism of various drugs and natural substances. This affects medications for heart conditions, mental health, cholesterol, and allergies. The same applies to foods, herbs, and natural supplements. Combining them can enhance healing due to the integrated power of their active components. Such is the case with various medicinal mushrooms in general, and Maitake in particular. Various papers were written on its ability, when combined with other compounds and substances, to alleviate a plethora of health conditions and diseases, such as blood pressure, prostate and bladder cancer, and HIV/Aids. (Halpern, 2007, p. 39)

7 Cordyceps *(Cordyceps Militaris)*

Although this thesis commissioner grows Cordyceps of the species *Militaris*, the following chapter encompasses information both of the species *Sinensis* and *Militaris.*

Cordyceps militaris is a fungus highly regarded for its medicinal properties and is already popular globally. While the availability of wild *C. militaris* is increasing in the recent years, large-scale cultivation of its fruiting bodies has been achieved successfully. (Lou et al., 2019)

Cordyceps is an edible fungus that grows by parasitizing other organisms. It contains a range of beneficial substances with metabolic properties. One of its key components, extracellular polysaccharide (EPS), has promising potential for preventing and treating certain diseases. The diverse structure and multiple bioactivities of EPS extracted from different parts of various Cordyceps species make it suitable for use in health foods and medicinal preparations. (Yang et al., 2020)

Cordyceps militaris has a unique appearance. It has a long body called a stroma, resembling a thin club or finger. The stroma can vary in size and has colours like bright orange or red. It produces structures called ascocarp that contain spores and can be clustered or branched. Cordyceps infects and grows inside insect larvae, like caterpillars, replacing their tissue. Eventually, the fungus releases spores through the stroma.

Figure 5. Grounded Cordyceps, as sold by “Nordic Mushrooms” for supplemental usage.

7.1 Historical Use

In the previous decade, *Cordyceps* was not considered as extensively studied for its bioactivity as other traditional Chinese medicines such as *Ganoderma*. For centuries, the combination of this fungus and deceased insects has been utilized as a traditional Chinese remedy. Its efficacy has been associated with the concept of Yin and Yang in Chinese philosophy. However, this can't probably be aligned with scientific principles. A significant body of literature exists, encompassing both scientific works and popular beliefs, including exaggerated claims. *Cordyceps sinensis* is the most extensively investigated species, followed by *Cordyceps militaris*. (Paterson, 2008)

7.2 Cultivation Methods

While over 400 species of *Cordyceps* have been described, only about 36 species have been artificially cultivated to produce fruiting bodies. Among these, *C. militaris* is the primary species commercially cultivated due to its strong pharmaceutical effects and short production time. It is grown in liquid media to harvest mycelia and on solid media to induce fruiting bodies. Although the abovementioned cultivation techniques have been optimized, large-scale production of *C. militaris* fruiting bodies currently relies on solid media made of artificial substrates or insects like silkworms. However, due to the high cost of insect cultivation, artificial media with rice as the main ingredient is predominantly used. (Shrestha et al., 2012, pp. 605, 607) "Kääpä" outsources its *Cordyceps militaris* substrates, and it is done on grain other than rice, such as barley.

The substrates used for industrial cultivation of *C. militaris* in China have recently been reviewed, including media for stock culture, pre-culture spawn, and spawn. The ingredients vary depending on the purpose and the company. Although the cost of cultivating insects has decreased significantly, fruiting bodies grown on insects are twice as expensive to produce compared to those cultivated in artificial media. However, when compared to the extremely high price of *Ophiocordyceps. sinensis*, the price of *C. militaris* is considered affordable. (Shrestha et al., 2012, pp. 605, 607)

In the cultivation of *C. militaris* fruiting bodies, four important growth periods are identified: mycelial culture, pigment induction, stromata stimulation, and fruiting body production. Successful cultivation requires precise control of temperature, humidity, and light. *C. militaris* fruiting bodies and mycelia are used in health products and drugs. In China, various health products such as oral liquids, capsules, wines, vinegars, teas, yogurt, and soy sauce containing *C. militaris* can be found. Cultures of *C. militaris* are also utilized in the production of drugs for kidney and lung function maintenance, anti-aging, sleep regulation, and chronic bronchitis. (Shrestha et al., 2012, pp. 605, 607)

7.3 Medicinal Effect

Cordyceps contains a diverse range of natural substances, including nucleosides, proteins, cyclic peptides, sterols, polyamines, and polysaccharides. Among these, the key bioactive compounds in Cordyceps are the nucleosides cordycepin and its analogues, polysaccharides, and sterols. These compounds are associated with various pharmacological effects, including anticancer, immunomodulatory, hypoglycaemic, and aphrodisiac properties. The majority of the reported health benefits of *Cordyceps* come from animal experiments conducted in vitro and in vivo, along with a limited number of clinical trials. *Cordyceps* is generally considered safe for human use and shows potential as an adjunctive therapy (a therapy that is given in addition to the primary or initial therapy to maximize its effectiveness) for conditions such as diabetes, cancer, and renal failure. (Prasain, 2013)

Cordyceps Militaris and Cancer

The need for an efficient and effective treatment for cancer, which is currently one of the leading causes of mortality worldwide, is still in high demand. The chemical Cordycepin (3′-deoxyadenosine) from *C. militaris* has an anti-tumour (or anti-cancer) action. Insights from research on this specific chemical component of the disease's treatment seem promising. *C. militaris* aqueous (liquid) extract inhibits proliferation and triggers apoptosis (natural process where cells in our body die in an organized and controlled way to make room for new cells) in human lung cancer cells. (Shweta et al., 2023)

Cordyceps Sinensis and Asthma

In China, doctors often recommend *Cordyceps* for treating asthma. A study conducted by Beijing Medical University showed that *Cordyceps* had positive effects on asthma patients. The study involved 50 patients between the ages of 17 and 65 who had tried antibiotics and other Western medications in vain. Out of these, 32 patients received 3 grams of *Cordyceps* or 10 milligrams of the antihistamine astemizole for 10 days. The results showed that the *Cordyceps* group had an effective rate of 81.3%, with 10 patients showing improvement in their forced expiratory volume test scores, and an additional 16 patients experiencing a 20% increase in scores. In comparison, the antihistamine group had an effective rate of 61.3%, with seven patients not responding to treatment. The *Cordyceps* group showed improvement in just five days on average, while it took nine days for cough to subside in the antihistamine group. (Halpern, 2007, pp. 78–79)

Cordyceps Sinensis and Diabetes

A study examined the effects *of Cordyceps sinensis*, taurine, and their combination compared to glibenclamide (a diabetes medication) on diabetes. Diabetic rats were given these substances orally for 3 weeks, and their effects were observed in vivo and in vitro. *Cordyceps*, taurine, and their combination reduced blood glucose, fructosamine, cholesterol, and triglyceride levels, as well as insulin resistance and pancreatic malondialdehyde content. *Cordyceps* increased insulin, antioxidant levels, HDL-cholesterol, and improved pancreatic function. Although taurine did not significantly elevate pancreatic antioxidant levels, both *Cordyceps* and taurine were more effective than glibenclamide as a stand-alone treatment in reducing insulin resistance and had strong antioxidant properties. (El Ashry et al., 2012) The usefulness of combined substances and synergy is discussed broadly in the Maitake chapter.

8 Turkey Tail *(Trametes Versicolor)*

Turkey tail is a type of fungus that grows on dead logs all around the world. Scientists call it *Trametes versicolor*, but it has other names like *Coriolus versicolor*. In traditional Chinese medicine, it's known as Yun Zhi, and in Japan, it's called Kawaratake or "roof tile fungus." The name "turkey tail" comes from its rings of brown and tan, which look like the feathers on a turkey's tail. There are other types of *Trametes* fungi that look similar to turkey tail, and in China, they use another species *called Trametes robiniophila Murr*, also known as Huaier. (PDQ Integrative, Alternative, and Complementary Therapies Editorial Board, 2002)

A distinguishable compound in *Trametes versicolor* called Krestin, which is used to make a medicine that can fight against cancer. In his book, Halpern (2007, p.99) claims that even though the United States Food and Drug Administration (FDA) hasn't approved Krestin yet, it was very popular and sold a lot in Japan during the 1980s. The Japanese government's Health and Welfare Ministry, which is the Japanese equivalent to the FDA, was the first to say that Krestin is a good medicine for cancer. In Japan, all healthcare plans assist people with buying Krestin in cases of need.

Turkey tail mushroom (davey.com)

8.1 Historical Use

For centuries, *Trametes versicolor* has been utilized by the Japanese as a traditional solution for cancer. In the realm of traditional Chinese medicine, *Trametes versicolor* is employed in the management of respiratory infections, excessive mucus, and hepatitis. The mushroom held deep significance among the ancient Taoists due to its growth on pine trees. Since pines retain their lush foliage year-round, Taoist priests associated the mushroom with the enduring strength of the pine tree. Taoists believed that *Trametes versicolor* absorbed yang energy from the pine tree's roots, and thus recommended it for patients experiencing deficiencies in their yang energy. (Halpern, 2007, pp. 99–100)

8.2 Cultivation Methods

In nature, *Trametes versicolor* grows in temperate forests worldwide, including the United States. It exhibits a preference for growing on deceased logs and demonstrates the ability to utilize various tree species as a food source. (Halpern, 2007, p. 99).

When being used for medicinal purposes, *Trametes versicolor* is commonly processed for consumption in powdered form extracted from the fermented substrate it grows on and its fungal mycelium. Different substrates are in use; “Kääpä” is using a combination of overnight soaked barley and vermiculite, while Benson et al. (2019), for instance, examined *Trametes versicolor* cultured on rice flour.

8.3 Medicinal Effect

Coriolus versicolor is widely prescribed for the treatment of cancer and infection in China, and prophylaxis (preventive measures taken to avoid or reduce the risk of a particular disease or infection).

Over the past few years, it has been widely demonstrated both clinically and pre-clinically that water-based extracts from *Coriolus versicolor* shows a wide array of biological activities, including suppression of cancer growth and stimulatory effects on different immune cells.

The growing popularity of water based *Coriolus versicolor* extracts as a supplementary medical method to conventional cancer therapies has generated significant commercial interest in developing these extracts into consistent and potent oral products. As for now, there has been adequate scientific evidence to support the feasibility of developing at least some of these constituents into a proof-based immunomodulatory agent. (Chu et al., 2002)

Krestin, PSK, and Cancer

Polysaccharide K (PSK) is a compound extracted from *Coriolus versicolor*, which has been extensively studied. It is made up of polysaccharides (62%) and proteins (38%). PSK has been shown to have no major impact on the immune system under normal conditions but can restore immune function in patients with cancer or after chemotherapy. It has been tested on different types of human cancers with some success. (Halpern, 2007, pp. 103–105)

When PSK is directly injected into tumours, it causes inflammation and kills abnormal cells. Local administration of PSK is more effective than systemic use. PSK also activates the expression of certain cytokines, which stimulate the immune system to fight against tumours. (Halpern, 2007, pp. 103–105)

Studies have demonstrated that PSK can improve survival rates in lung, breast, stomach, and oesophageal cancer patients when combined with chemotherapy. However, there have been conflicting results in some trials, which may be influenced by genetic factors. (Halpern, 2007, pp. 103–105)

PSK is considered safe with minimal side effects. It has also shown potential benefits in reducing LDL cholesterol and has antiviral effects. Although further research is needed, PSK holds promise as an adjunctive treatment for cancer and may continue to be used in the future. (Halpern, 2007, pp. 103–105)

Halpern (2007, p. 103) summarized Krestin's trajectory accurately in his book: "…Krestin came under fire beginning in the late 1980s at several medical conventions, where doctors questioned its effectiveness. The substance, it seemed, had been overhyped. The Health and Welfare Ministry in Japan now instructs doctors to use Krestin only as an adjunct to chemotherapy or radiotherapy. The drug by itself is not supposed to be used as a treatment for cancer. PSK can raise survival rates in cancer patients and prolong their lives. Moreover, the substance is nontoxic. Because the risk to patients of taking PSK appears minimal and the rewards are many, PSK is likely to be an aid in fighting cancer for years to come. PSK also causes decreases in LDL ("bad") cholesterol in patients with high levels of blood circulating fat. It was found to have an antiviral effect on HIV and cytomegalovirus."

9 Chaga (*Inonotus obliquus)*

Although "Kääpä" producing both *Pohjola* and *Napsu* strains, this segment deals with *Inonotus obliquus* species in general.

Inonotus obliquus, a member of the Hymenochaetaceae family, is a fungus that parasitizes plants and has a black-brown color. This mushroom has several common and regional names, likely derived from the Russian word "Chaga." Chaga is an edible herbal mushroom extensively distributed in the temperate to frigid regions of the Northern hemisphere, especially the Baltic and Siberian areas. (Peng & Shahidi, 2020) The genus *Inonotus* is found in North America, Asia, and Europe, with approximately 100 species. In Europe, there are only four species in this genus, including *I. obliquus*. Although widely distributed in North America, Asia, and Europe, I. obliquus is listed as a partly protected mushroom species in several countries. (Szychowski et al., 2021)

I. obliquus primarily parasitizes trees, causing the decay of live trunks. It has been observed on various tree species such as beech, maple, alder, rowan, hornbeam, poplar, willow, plane-tree, chestnut, oak, ash, and walnut. However, the main hosts for *I. obliquus* are different species of birch. (Szychowski et al., 2021)

The fungus infects trees aged approximately 30-50 years through wounds in the bark and can continue growing on the trunk for another 30-80 years. A few years after penetrating the live tree trunk, it produces sclerotia, which are vegetative or asexual fruiting bodies. These sclerotia have a lumpy irregular shape, cracked surface, and black-brown color. Inside the sclerotium, there is a dense mycelium that is rust-brown with yellow veins. The sclerotia grow slowly, reaching a diameter of over 10 cm in 10-15 years. On older trees, these growths can exceed 50 cm in diameter. After many years, the host tree dies, and the annual fruiting bodies of the sexual stage appear. Fruiting bodies of this stage develop during the warm season in areas with advanced rot. They rarely grow on live trees. These fruiting bodies can be quite large, reaching lengths of 3-4 m and widths of up to 50 cm. Insects, along with wind, are assumed to be the primary vectors for spreading the spores of *I. obliquus*, and they quickly consume these fruiting bodies. (Szychowski et al., 2021)

Figure 6. Chaga conk (Kääpä Biotech website, 2023)

9.1 Historical Use

(Szychowski et al. (2021) described in detail the historical use of Chaga mushroom that can be traced back to the 12th century in Eastern Europe. Historical records mention its healing properties in treating lip tumour among Kiev royalties. Traditional medicine in Siberia also utilized *I. obliquus* for various conditions, capitalizing on its antiparasitic, anti-tuberculosis, anti-inflammatory, and gastrointestinal benefits. It was commonly recommended for heart and liver diseases. Infusions, inhalations, and aqueous solutions were the primary forms of administration, while antiseptic soaps containing *I. obliquus* were formulated for external use. During the mid-20th century, Chaga infusions even served as a tea substitute in Siberia. Early attention was directed towards exploring the potential antitumor or supportive effects of *I. obliquus* in cancer treatment, especially before the advent of modern oncology. Notably, such references can be found in popular literature, including Aleksander Solzhenitsyn's book "Cancer Ward". (Solzhenitsyn, 1968/2003)

In Asia (Korea, China, and Japan), the Baltic countries and Russia, extracts derived from Chaga mushroom have been used due to their beneficial influence on the heart function, plasma lipid system, and their anti-inflammatory, antibacterial, and anti-cancer activities. Moreover, the antioxidant activity exhibited by *I. obliquus* holds significance in the prevention of civilized diseases associated with free radicals, including atherosclerosis, cancer, diabetes, accelerated aging, and degenerative disorders of the central nervous system. Research has also demonstrated the inhibitory effects of Chaga extracts on the replication of hepatitis C virus (HCV) and HIV. (Szychowski et al., 2021)

9.2 Cultivation Methods

Sun et al (2011) claims that In Russia, Poland, and most of the Baltic countries, *Inonotus obliquus* has long been utilized as a traditional folk remedy for addressing conditions such as cancers, cardiovascular disease, and diabetes. However, the availability of this fungus in its natural reserves has become scarce, nearing depletion.

The Chaga mushroom exclusively thrives on living trees, and as of now, it is solely harvested from its natural habitat, without any artificial cultivation methods in place (Ka et al., 2017). As mentioned, Chaga grows very slowly, and Ka et al (2017) summarizing their efforts in an experiment they have performed: "…We artificially cultivated chaga mushrooms by inoculating its sawdust spawns on Betula platyphylla var. japonica (Japanese white birch) in

2007 and monitored mushroom growth on the inoculated trees for 9 years. The mushrooms grew less than 1 cm per year, with the largest mushroom growing up to 9 cm in the 9 years of study".

9.3 Medicinal Effect

Chaga has been extensively investigated for its pharmacologically active compounds that have a positive impact on human health. The bioactivity of *I. obliquus* primarily stems from the presence of various polysaccharides composed of sugars such as rhamnose, arabinose, xylose, mannose, glucose, and galactose. Furthermore, numerous studies conducted in the past decade have highlighted the diverse biological activities exhibited by *I. obliquus*, including its anticancer, antioxidation, anti-inflammatory, antidiabetic, and immunomodulatory properties. In particular, several research endeavours have also indicated the favourable safety profile of *I. obliquus* in the context of disease treatment, with minimal or no observed side effects.

Chaga and non-alcoholic fatty liver disease

Currently, a common liver disease called non-alcoholic fatty liver disease (NAFLD) is prevalent worldwide. However, there is a lack of specific medications for treating NAFLD. *Inonotus obliquus*, known for its preventive effects against metabolic syndrome-related conditions like diabetes and hypertension, has been studied for its potential impact on NAFLD. Researchers aimed to investigate whether *Inonotus obliquus* can inhibit NAFLD, identify the active components in it, and understand the mechanisms behind its beneficial effects. The findings revealed that *Inonotus obliquus* and its extracts, including inotodiol (Ino), lanosterol (Lan), and trametenolic acid (TA), significantly improved lipid accumulation in the livers of mice with NAFLD or liver cells treated with a specific substance. Furthermore, the study showed that *Inonotus obliquus* and its components reduced the activity of genes responsible for fat production while increasing the activity of genes related to fat metabolism regulation. In particular, the inhibitory effects of Inotodiol on fat deposition in liver cells were reversed when a specific substance called guggulsterone (GS) was introduced, which blocks a particular pathway. In summary, *Inonotus obliquus* and its components alleviate fat accumulation in the liver by hindering fat production. The positive effects of Inotodiol, a bioactive compound found in *Inonotus obliquus*, are achieved by regulating a specific pathway. These findings indicate that *Inonotus obliquus* and Inotodiol hold potential as promising therapeutic options for NAFLD. (Peng et al., 2022)

Chaga and Cancer

The Chaga mushroom has a history of use in traditional medicine for cancer treatment. A study (Chung et al., 2010) investigated the potential anticancer effects of specific compounds derived from the Chaga mushroom *(Inonotus obliquus*) in mice with Sarcoma-180 *(murine Sarcoma cancer cell line)* cells and human carcinoma cells. The researchers tested the inhibitory effects of different subfractions of the Chaga mushroom on various human carcinoma cell lines in vitro. They then implanted Sarcoma-180 cells in mice and supplemented their diet with different doses of specific subfractions. All subfractions showed significant cytotoxic activity against the cancer cell lines in vitro. Subfraction 1 exhibited greater activity against certain cancer cell lines. In the in vivo results, subfraction 1 at specific concentrations significantly reduced tumour volume compared to the control. Subfractions 2 and 3 also inhibited tumour growth in mice with Sarcoma-180 cells. Subfraction 1 showed the highest inhibition of tumour growth, consistent with the in vitro findings. These results suggest that the compounds from the Chaga mushroom, specifically those found in subfractions, could have potential as natural anticancer ingredients in the food and pharmaceutical industry.

Breast cancer is a significant health issue due to its widespread occurrence (Anastasiadi et al., 2017). Standard treatments for breast cancer include surgery, chemotherapy, radiation therapy, and hormonal therapy, leading to a cure rate of 90% for early-stage cases (Wörmann, 2017).

With such high survival rates, there is growing interest in exploring complementary and alternative medicines as potential treatments for breast cancer. However, limited research exists on the effectiveness of these treatments, and many patients resort to them without adequate scientific information. It is important for breast cancer patients to exercise caution when considering complementary and alternative medicines due to the heterogeneous (refers to the diversity and variability that exists among cancer cells within a tumour or among different tumours in the same type of cancer) nature of breast cancers. (Buckner et al., 2018)

Chaga mushroom extract is a popular botanical supplement among cancer patients. (Buckner et al., 2018) Recent reports have highlighted its use in a breast cancer patient with triple-negative breast cancer (Santoni et al., 2018). TNBC (Triple Negative Breast Cancer), is a specific subtype of breast cancer characterized by the absence of three receptors known to fuel the growth of most breast cancers: Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). In light of this, A study (Lee et al., 2021) aimed to explore the potential anticancer effects of Chaga mushrooms on various types of breast cancer cells. Additionally, we sought to investigate the molecular mechanism underlying these effects.

This concludes the literature review chapter. There are several other mushrooms the commissioner is growing, treating, and developing, but the writer decided to deal solely with the prominent ones. The next chapter, “Materials and Methods”, as well as the following chapters will be discussing only Reishi (*Ganoderma Lucidum)*, as part of the experiment in discussion.

10 Materials and Methods *(Ganoderma Lucidum)*

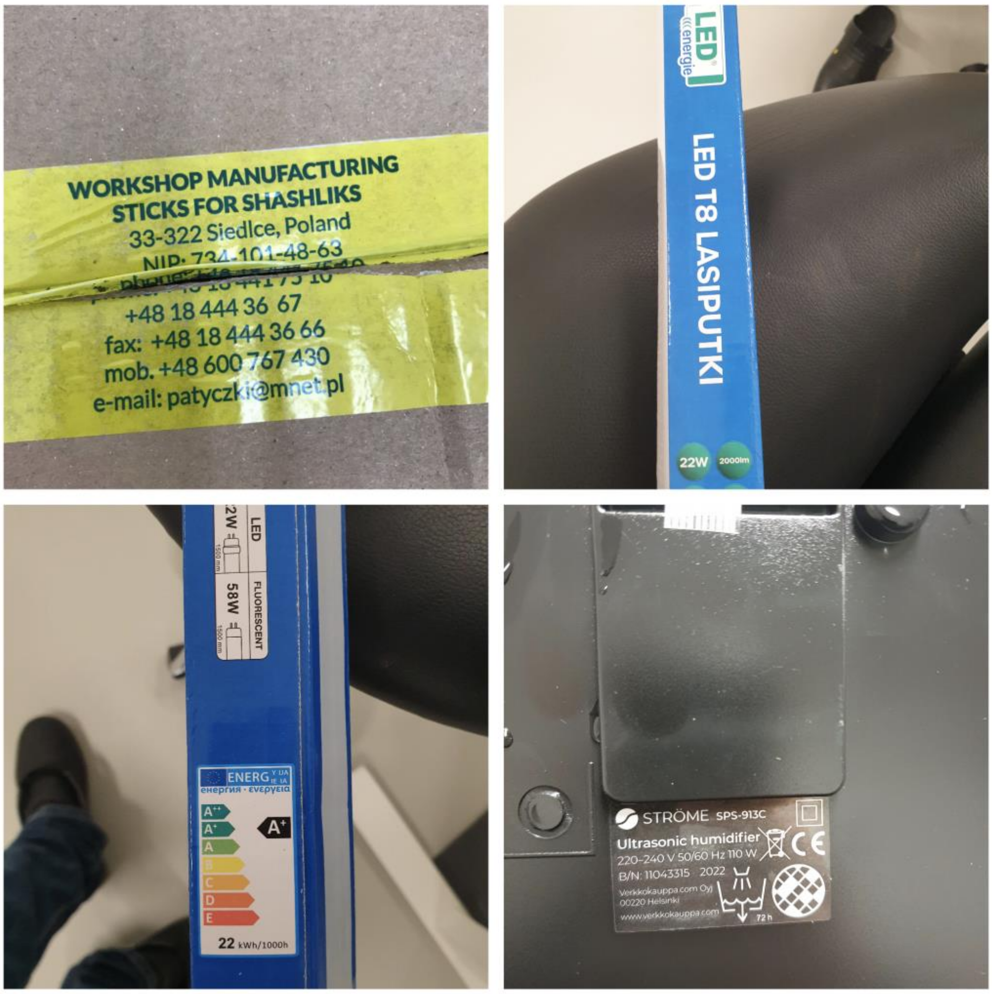
As part of the upscaling efforts of Kääpä Biotech (which are almost completed as of this writing), the commissioner was trying to establish viable methods for growing *Ganoderma Lucidum* on a larger scale. The effort was towards finding ways which are more time efficient, and that would correlate with its current, limited work force. The following is a detailed report of all parts of the experiment (both preliminary and actual), but the final results that are brought upon relate solely to the actual experiment. That is mostly due to lack of interest by the commissioner, but also due to loss of some of the data of the preliminary experiment. Regardless, that does not affect the results or other possible outcome. A detailed activity log which described both experiments and other happenings that may affect is to be found in the appendix.

10.1 Materials

The experiment took place inside a designated room (size 3.5 meters X 6.8 meters) within the lab. The room was used for similar projects, including the preliminary experiment. The room was thoroughly cleaned before and after every experiment, the floors washed with soap and chlorine and shelves were sprayed with ethanol.

- Unicorn Bag (3TL, Dimensions: 56 x 20 cm)
- Spelt and sawdust substrate (2.28 kg, 40X15)
- Microsac boxes (Cover: 195 × 195 mm, Base: 185 × 185 mm, Height: 191 mm, Volume: 5000 ml)
- Greenhouse arched tent (3X2 meters)
- Shelving units (1 unit outside the tent, 1 inside, and 1 small unit inside the tent, covered with plastic sheet):
- Outside the tent: 1.77 meters, 8 shelves, 3 of them containing substrates – 20 substrates in total.
- Inside the tent, 2 units; 1: 1.77 meters, 8 shelves, all in use, 44 substrates in total.
- Inside the tent: A plastic covered unit, 91 cm, 5 shelves, all are in use with 4 substrates on each shelf.
- Plastic sheet
- Sensors
- for the tent: Carbon dioxide detector, unknown manufacturer.
- 2 “NetAtmo” sensors; 1 outside the tent, and one for “Tent tent”.
- Autoclave
- Skewers (beech wood, made in Poland)
- Markers (Used to mark different cutting methods of substrates)
- Hydrogen Peroxide (used as attempt to eliminate mold in an advanced stage of fruiting, probably towards its end)
- Mikkolan Sienituote substrate (initial weight:2.26 kg approx., unknown Reishi strain)

Figure 7. A selection of the equipment used in the experiment. Top left clockwise: Skewers for inoculation, LED lights, and humidifier.



10.2 Methods

The experiment was divided into several stages: First, the preliminary experiment took place, and was composed of two stages. In the first stage, 6 substrates were inoculated (all provided by Mikkolan Sienituote with Reishi strain of “Kääpä” inventory. A few days later, it has been decided to add 3 more substrates and inoculate them as described in the flow chart (Appendix 1). Despite not having enough research objects for the preliminary stage, it has been decided to utilize these substrates, also due to lack of time (the commissioner was already interested in initiating the experiment as part of research and development initiative), as well as to make use of the substrates that were left unutilized. During the preliminary experiment, substrates were color coded according to its cut: Green for a single hole, Blue for 3 holes in close proximity, and red for a diagonal, elongated slash. After close observation and conclusion of the preliminary experiment, it has been decided that the elongated slash is the most beneficial, hence all the substrates in the actual experiment were cut this way.

Figure 8. Tent sketch, prior to its establishment. Colour codes as follow: Green for a single hole, Blue for 3 holes in close proximity, and red for a diagonal, elongated slash.

The main experiment was initiated on the 15th of August 2023, almost 6 months after the beginning of the preliminary experiment, but in close proximity to the termination of it. 84 substrates, all inoculated by “Kääpä” strain (but processed by “Mikkolan Sienituote”) were received, partially miciliated (“Kääpä” uses this particular service by “Mikkolan Sienituote” with different kinds of mushrooms.

The writer decided to add the main events of the activity log, to assist the reader with a chronological understanding of the steps taken along the way, and also to enable a possible future attempt to mimic the experiment.

The following text, which describes the preparation, sterilization and inoculation of the substrates refers solely to the preliminary experiment that was made from start to finish by the writer and the lab team, unlike the actual experiment that was prepared, sterilized, and inoculated by “Mikkolan Sienituote”, using Kääpa Biotech strain.

Preparing sticks

The appropriate amount of birch sticks was weighed out. A standard batch of 500 sticks was weighed at 600 grams.

The sticks were placed in a container for soaking. The container was filled with hot water, enough so that the sticks were fully submerged and will remain submerged after absorbing the water. They were left to soak for approximately 18 hours.

After 18 hours, the sticks were drained so that no water remained.

Sterilizing

The soaked and drained sticks were loaded into the final container. Under normal conditions, this can be done using Unicorn bags or Microsac boxes. The boxes can hold 500 sticks when placed horizontally or 1000 sticks when placed vertically. The 10T Unicorn bags can hold 500 sticks when positioned horizontally, or the 3TL bags can hold 1000 sticks when positioned vertically. The box was prepared in a way that prevents the lid from sealing closed during the cooking process.

A pressure cooker was employed for sterilization. The bags were kept above the water line by a separating insert, and they were placed overlying in a 4–5-centimeter water level at the bottom of the cooker. A metal grate was placed above the top layer of the bags to prevent blocking the steam valve by swelling bags.

The lid was locked, the cooker was placed on a hotplate, and heating was initiated by setting the stove to high. Steam leakage has been observed, and the outlet was closed by attaching the regulator at 103.42 kPa. The sterilization time was counted from the moment the pressure gauge reaches 15psi, at which point the heat is also reduced.

Sterilization was carried out for 2.5 hours at 121 degrees Celsius and 103 kPa (kilopascals).

Once sterilization was completed, the oven was turned off, and the pressure decreases to 5psi. The cooker was then moved to the lab and allowed to decrease to 0 in front of the running HEPA filter. All surfaces were wiped with 70% ethanol. The bags were then unloaded and allowed to cool in front of the filter for same-day inoculation, but under different circumstances should be sealed and stored.

Inoculating sticks

Sterilized sticks can be inoculated with either grain spawn or sawdust spawn, and a combination was used in the discussed case.

The workstation was prepared by ensuring that the HEPA filter has been running for at least 15 minutes prior, and the table and scalpel holder were wiped with 70% alcohol.

Empty containers of sticks, as needed, were selected. These were sterilized and cooled to a temperature below 35 degrees Celsius at the centre. They were placed in front of the HEPA filter. The inoculum bag to be used for inoculating the substrate were selected. The spawn should be fully colonized, must not be older than two months, properly stored, and should show no signs of contamination. It was carefully checked for contamination, and then was shaken up to break apart clumps.

The substrate bag(s) or boxes were opened if needed, and the sides of the bag were lowered without touching the lip or inside. The pouring corner of the grain bag was wiped with alcohol. A hole was made in the corner, which is just big enough to pour from. Without touching or entering the substrate bag, the grain bag was held over it, with one hand at the hole to regulate the pour rate.

The inoculation rate is heavy, approximately 400g. Such an amount is to verify that skewers are inoculated properly inside the bags, for its peculiar shape doesn’t always allow some of the skewers to be fully covered or covered enough to promote successful inoculation within the substrates.

The bags or boxes were then sealed, starting with the spawn bag, with adequate amount of air inside. The substrate bags or boxes were shaken to ensure the even distribution of the spawn, and at least a couple are visible for growth monitoring. A label should be placed on the bag(s) by affixing a piece of duct tape and writing the species, strain, p-value, and today's date on the tape (or below), but these details were written directly on the substrate, and were also documented on internal lab documents, as required. The Inoculated sticks were placed on a shelf for colonization.

Cutting Methods

It is important to mention that all the 11 substrates of the preliminary experiment (before the addition of 109 substrates provided by "Mikkolan Sienituote") were sorted in groups and inoculate from different angles on the substrate. The substrates were divided into 2 groups; Either 3 or 5 holes were punched into the substrates using the miciliated skewer, and either it was done from top part of the substrates, its sides, or the "axis" (term we coined), referring to both the short corners of the substrates. The following are the dates and methods of inoculation:

12th of April: A1: 3 holes/ A2: 5 holes, Side (from the tips) inoculation

B1: 3 holes/ B2: 5 holes, Side inoculation

C1: 3 holes/ C2: 5 holes, Upper inoculation, crossed.

18th of April: A3 – 3 holes from the tips

B3 – 3 holes from the sides, B4 5 holes from the sides

C3 – 3 holes on top, C4- 5 holes on top

Figure 9. Example of side inoculation. The skewers that got broken (on purpose) are visible on the sides, 3 holes on B1 and 5 holes on B2.

10.3 Experimental Errors

Several errors occurred during both of the experiments. These events were included in the original activity log and can be handed for further investigation in case of interest.

There are several challenges which mushroom producers are facing, and especially in the organic mushroom field, where fungicides are not to be used. (Grogan, 2008, p. 1) Beyond the normal hygiene regulations that are accepted in Kääpä Biotech lab environment, such as designated shoe changing when moving from one room to the other, and different coats that are to be worn when performing different tasks in different parts of the lab. There was a constant usage of nitrile of latex gloves, face mask (fabric or hardened plastic, depends on the need), and constant usage of ethanol to be sprayed upon the gloves. Despite the attempts to maintain a sterile environment that even included the usage of hydrogen peroxide, both the preliminary and actual experiments batches ended to contain mold. The grow room where both of experiments took part was cleaned and sterilized thoroughly in between both experiments, which did not help, for mold was still present. Reasons for that will be discussed broadly in the discussion chapter of this report.

Another error that in all likelihood did not affect the results but certainly sabotaged the monitoring of the experiments is the usage of sensory devices to deliver results on a daily basis. The main cause was dying or dead batteries that had to be replaced.

At the end of the preliminary experiment, it was clear that the experiments should be adjusted and take the form of a qualitative, rather than a quantitative one. Firstly, many aspects that were quite challenging to measure, such as the antlers length, for the fruiting body formed a very unique and clumpy shape that made it difficult to decide where to measure form. The dispersion of the fruiting body was not uniform, hence such results are not to be included in the report. Unfortunately, even weighing of the preliminary experiment biomass wasn’t possible due to heavy contamination and the lab manager’s instruction to get rid of the biomass immediately did not allow the writer to weigh the biomass. Thankfully, that was not the case in the actual experiment, and biomass weigh is available and will be presented in the results chapter.

11 Results + Discussion

Other than medium composition, several studies and research describe different factors as contributing to biomass. Such factors are, but not limited to initial medium pH, aeration rate, inoculum density and more (Simonic et al., 2008). Growth media, and even carbon and nitrogen sources are also taken into consideration when discussing the optimization of Reishi growth in artificial setting (Jayasinghe et al., 2018). These were omitted due to the nature of the thesis, but not solely; it was made clear that such factors are irrelevant for the purpose of this R&D project, and only factors such as temperature, humidity, and CO_2 levels were required and documented, as will be shown in this chapter.

Measures were monitored in two ways: During the preliminary experiment, the team was only monitoring progression in a sensory manner, meaning progression was only measured by looks of the substrate and fungi or smell in the tent, and tunings were made accordingly. Since it was the first time such a thing was attempted by the commissioner, and work overload during springtime, it was decided not to document any measures in that regard.

Results describe merely the plausibility of growing *Ganoderma lucidum* artificially in lab conditions, and for the naked eye, it might seem like not enough measures were made, which might be true to an extent. Unfortunately, mold that appeared at the end of both preliminary and actual experiment did not allow the team to measure biomass properly (at least at the preliminary experiment), for there was need to evacuate the fruiting body as soon as possible outside of the lab. During the actual experiment, mold appeared as well, although in quantities that did not allow future utilization for the commissioner needs, but certainly allowed for weighing the biomass. These results are also brought in this chapter.

<u>CO_2Temperature, and Humidity levels</u>

During mycelium development preceding perforation of substrates, carbon dioxide (CO_2) concentrations elevate significantly. Subsequently, mycelium ceases growth due to insufficient oxygen availability to sustain metabolic processes with the remaining resources. Upon incision, the mycelium is no longer oxygen-limited, leading to an accelerated consumption of readily available nutrients, thereby causing a sudden spike in CO_2 levels. This rapid growth is pivotal as it serves as a trigger for subsequent developmental stages. (Heikki Kiheri, personal communication, Scientific director at Kääpä Biotech, December 2023)

For the elongated antler form, it is imperative to maintain CO_2 levels below 2000 ppm. Achieving this within the lab's current air exchange system proves challenging due to a lack of mechanisms and potential hazards to the lab team. Despite these constraints, a compromise was reached, recognizing the importance of obtaining data. Surprisingly, the initial test conducted by the lab team, serving as a preliminary experiment, yielded positive results. Subsequent attempts were less successful, attributed to an inadequate supply of growing substrates leading to insufficient CO_2 levels. (Heikki Kiheri, personal communication, Scientific director at Kääpä Biotech, December 2023)

In response, the team opted to maintain a temperature of 25 degrees Celsius during the growth period based on successful outcomes observed in a previous Reishi growing experiment utilizing Unicorn bags.

The following are average measures of CO_2, temperature and humidity from the actual experiments, divided setting (“Room”, “Tent”, and “Tent tent”), showing all (three) 3 months average.

Table 1. CO_2, humidity, and temperature of all 3 settings of experiment.

Settings	CO_2 (3 months average)	Humidity (3 months average)	Temperature (3 months average)
“Room”	459.652	52.66%	21.06
“Tent”	1456.562	69.57%	22.3
“Tent tent”	3753.996	86.50%	24.32

- Data was not collected on "Tent tent” during October, after sensor battery (probably) died. The team decided not the life the plastic cover regardless, in order to optimize conditions and try to imitate as natural settings as possible, hence data is lacking. The data being brought is from months August-September only.

- The writer also decided to consider negligible some unusual events during measuring. For instance, irregular measuring time (i.e., 26th of August on “Tent tent”, measures were taken at 11:15 or 22:15 (instead of usual 7:15 am). The team realized it does not affect the results, but worth mentioning as for keeping the report as accurate as possible.

- It is quite obvious that there is a clear growth in all measures, moving from “Room” (the biggest space of experiment) to “Tent“ and “Tent tent” (the smallest, most dense space). The only exception is the average temperature in “Tent tent”, but that is only due to missing measure in October, after sensor stopped working (as mentioned in the first point).

<u>Moldy substrates during the preliminary experiment</u>

- The laboratory team is leading a zero-tolerance policy in regards of mold, and safety measures are being taken before entering the lab, during working hours and after leaving the lab. Despite all of that, mold did appear on certain substrates (perhaps due to the usage of the space in discussion for previous project, the details of which the writer cannot disclose). During the preliminary experiment, the following substrates found moldy and were discarded immediately to prevent infestation and jeopardize the experiment: Substrates B1, A1, and C4. A small moldy spot was removed with a scalpel from substrate A2 but was not totally discarded.

Figure 10. Substrate A1 infected with green mold at its edge.

Moldy substrates during the actual experiment

Moldy substrates appeared during the actual experiment as well. On the 18th of August 9 such substrates were detected and removed from the tent. Later on, on the 25th of September 12 such substrates were detected and removed from tent.

Unusual parameters during the experiment

This part describes several unusual events during the experiment. Although few unusual (deviation from norm) events took place, the writer decided to leave only the events that are worth mentioning, for its occurrence might have affected the results.

- On the 23rd of May, during the preliminary experiment and after adding 109 Inoculated Reishi substrates from “Mikkolan Sienituote”, the highest level of CO_2 was measured in the tent: 2393 ppm. It would be fair to acknowledge that during the prior day, when addition took place, another high measure had been taken: 1270 ppm.

Figure 11. CO_2 measure on the 23rd of May

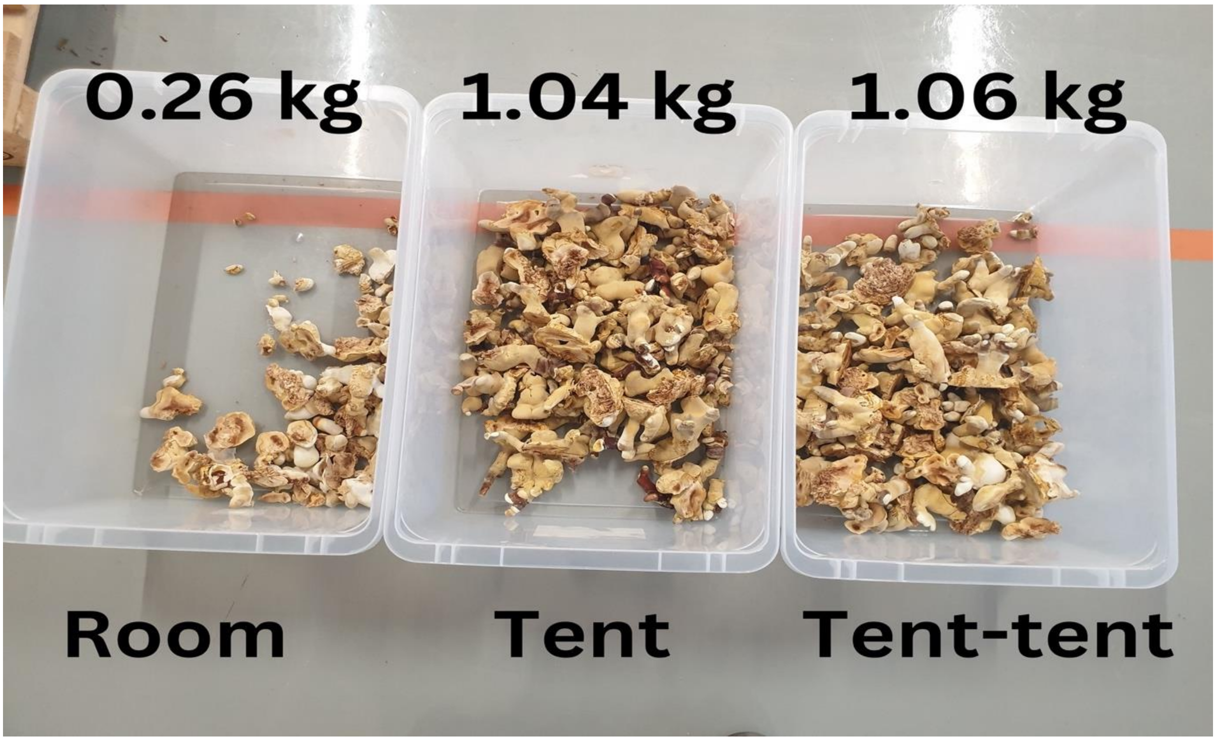
- On the 15th of August, during the actual experiment, another CO_2 record high took place: 4095 ppm in the "Tent tent" (which is the most enclosed and untouched part inside the tent). This happened few hours after cutting the substrates and setting up the tent.

Biomass measures of actual experiment

Biomass of the actual experiment measured as following, after harvested and removed off tent while still contain mold.

- "Tent tent": 1.06 kg
- "Tent": 1.04 kg
- "Room": 0.26 kg

Figure 12. Actual experiment biomass. From right to left: "Tent tent", "Tent", and "Room."



Underdevelopment of fully matured antler form

Unlike the preliminary experiment, where antlers developed to the desired, elongated shape with the right colour gradation (as seen on Figure 2), the actual experiment did not produce similar results.

Amongst several plausible reasons, the lab team assumed that it is possible that antlers did not form properly due to a different inoculation method in “Mikkolan Sienituote” facility; The lab team used inoculated skewers that were inserted from different places through the substrates, using various amounts of skewers (3 or 5). Perhaps the method of preparing the inoculum were faulty, a factor that might affect the development of the final fruiting body.

It is noteworthy that the team also knew beforehand that this Reishi is mainly for R and D purposes, hence the tent setting (substrates too close to each other) will eventually create weird, probably deformed shape (at least in the narrowest space of all, “Tent tent”, where fruiting bodies got mashed together into a chunk, but this as well should not affect its progress into a fully mature antler shape.

The information that was gathered during the experiment is very valuable to the commissioner and the company as a whole. Although utilization of fruiting bodies was not possible due to contamination, the success in understanding the conditions that enabling the production of *Ganoderma Lucidum* partially satisfies the process of upscaling within the company. The understanding of humidity, temperature and CO_2 levels needed, especially for an experiment that is being done for the first time, is not taken for granted. Perhaps in the future, usage of a shelf units covered in tightly controlled tent would not only produce superb fruiting bodies, but such that would be utilized by the commissioner and be considered as another flagship product on a mass scale, just like other nutraceuticals produced by Kääpä Biotech.

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Appendix 1. Chronological flow diagram

The following is a selected events log which the writer used in order to describe every event in the growing room in which where the experiments (preliminary and actual) took place. For obvious reasons, this is not an exhaustive list, for the writer included only the important events that has an effect on the experiments.

Date	Event
29/03/2023	Skewers moved into unicorn bags after being soaked for 48 hours, and were sterilized in an autoclave for 2.5 hours.
30/03/2023	Skewers were inoculated; 2 bags, 50 skewers in each.
03/04/2023	Mycelium started developing in skwers bag.
12/04/2023	Substrates were ready to be inoculated. I thought about using 4 of the "sausages"; 2 will be inoculated from the sides, while the other 2 will be inoculated from the upper part. There are 10 skewers that aren't buried properly under the mycelium, which leaves 40 in each bag, hence the calculation. Tent was set up, cleaned, and sterilized with chlorine. Humidifier installed and set to 70%. 12 LED lights, working in 12/12 cycle, to imitate natural conditions, aligned with natural cycle – Light during the day, darkened during the night. Sausages were inoculated; A1: 3 holes/ A2: 5 holes, Side (from the tips) inoculation B1: 3 holes/ B2: 5 holes, Side inoculation C1: 3 holes/ C2: 5 holes, Upper inoculation, crossed.
14/04/2023	Lion’s Mane inoculated substrates added to tent. CO2 sensor was put in the tent.
17/04/2023	Mycelium started to appear around the place of inoculation sticks. CO2 meter measured around 1000 ppm
18/04/2023	5 new substrates added to the experiment: A3 – 3 holes from the axis B3 – 3 holes from the sides, B4 5 holes from the sides C3 – 3 holes on top, C4- 5 holes on top. All holes of new added substrates are covered with filter stickers.
20/04/2023	Started monitoring CO2 level in the tent: Twice a day, morning and close to the end of the shift.
11/05/2023	5 substrates (B2, A2, A3, C1 and C2) went into primordial stage. Cross slashed at the axis and water routine (once a day) started.
22/05/2023	109 reishi inoculated substrates were added to the tent. CO2 level went substantially up (1270 PPM). Preliminary experiment Reishi demonstratred well fruition.
23/05/2023	2393 ppm CO2 were measured
31/05/2023	New substrates were divided into 3 groups according to their cut: diagonal, one small hole, and 3 holes. Watering started. Tent re-organization started.
01/06/2023	Tent was re-organized; two new shelving system were assembled (5 shelves each), one covered using a plastic sheet. Coloring system was decided upon. 2 large shelving system (8 shelves) assembled and put outside of the tent. Sensor was put in the covered tent.
06/06/2023	5 weeks delay of the actual experiment casued due to shortage of substrates supply.

14/06/2023:	Preliminary experimental Reishishowed good fruition. Mikkolan Sienituote Reishi started fruiting sporadically.
15/06/2023	Reishi watering stopped. Decided to stop removing the plastic from "Tent tent" after fruiting started
30/06/2023	noticed that substrates with diagonal cut are fruiting better outside of the tent. In the tent, it seems like all cutting methods are pretty much the same in fruiting mass.
10/07/2023	Few moldy spots around several substrates were detected in the tent. Decided to spray with Hydrogen Peroxide.
12/07/2023	Sporangium noticed.
13/07/2023	84 substrates inoculated with "Kääpä" strain delivered today. Substrates were put into the big grow room. Decided to wait until the substrate gets fully myceliated and to get pierced only then.
02/08/2023	Preliminary experiment ended officially. Data gathering.
15/08/2023	(Main experiment began) Reishi room was cleaned to ensure no mold remainders. Substrates were pierced, all sensors were calibrated. CO_2 record in Tent-tent: 4095 ppm, at 19:45 (a few hours after making the holes and setting up the tent).
29/08/2023	Watering stopped.
30/08/2023	Hydrogen peroxide supply was renewed, started using again to take care of moldy spots.
05/09/2023	Reishi already started to show antler form. Moldy spots are treated with H_2O_2.
07/09/2023	Moldy spots showed to be improved, very few around and most seems like were consumed by the Reishi.
09/10/2023	Sporangium noticed.
18/10/2023	Experiment officially ended.