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„Fungal community in Austrian Vorarlberger Bergkäse during ripening"

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2. Acknowledgment

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3. Abstract

Vorarlberger Bergkäse (VB) is an artisanal raw milk washed-rind hard cheese (manufactured in Western Austria) without adding external ripening cultures and has a protected designation of origin (PDO). The aim of this study was to identify cheese-associated eukaryotes (yeast and filamentous fungi) present in the VB cheese rinds during ripening process. Cheese rind samples (n=200) were taken from two cheese producing plants in Austria at the first day of production and after 14, 30, 90 and 160 days of ripening. High-throughput gene-amplicon sequencing (Illumina MiSeq) targeting the ITS2, real-time quantitative PCR (qPCR), targeting 18S rRNA gene for fungi and cultivation approaches were used to determine the diversity and abundance of fungi in VB cheese rinds. The qPCR results show that fungi are abundant on VB-rinds throughout ripening in both plants. Fungi decreased significantly during the first 14 days of ripening ($p<0.001$) in both plants, but then increased significantly ($p<0.001$) towards 90 days in one plant.

The qualitative investigation of fungal communities on VB rinds throughout ripening gave the first preliminary insight into the VB-fungal profile in cheese rinds. A comparison between different ripening points showed *Saccharomycetales family Incertae sedis* ASVs (genus *Candida,* osmotolerant ascomycetes yeast) to be most dominant during the first 14 days, while its relative abundance decreased afterwards. On the contrary, *Microascaceae* (genus *Scopularopsis*) were lower at day 0 and 14 and increased thereafter in both plants. *Debaryomycetaceae* (genus *Debaryomyces* and *Yamadazyma*) increased after the first day of production and subsequently decreased during ripening.

These new findings enable us to understand the VB cheese-making process better and might allow the processing- and ripening conditions to be improved to enhance the quality of the product.

3.1. Abstract German

Vorarlberger Bergkäse ist ein Rohmilch Hartkäse mit gewaschener Rinde, der traditionellerweise in Westösterreich hergestellt wird und eine geschützte Herkunftsbezeichnung besitzt (PDO). Dabei wird auf den Einsatz externer Reifekulturen verzichtet. Während der Reifung befinden sich unterschiedliche Hefe- und Fadenpilze auf der Käserinde. Das Ziel dieser Studie ist es, diese Pilze zu identifizieren. Hierzu wurden am Tag der Produktion und nach 14, 30, 90 und 160 Tagen Proben (n=200) aus zwei Käsereien in Österreich entnommen. Mittels Sequenzierungen (Illumina MiSeq), quantitativer Echtzeit-PCR (qPCR) und Kultivierungsansätzen wurden Diversität und Abundanz der Pilze auf der VB-Käserinde bestimmt. Die Ergebnisse der qPCR zeigen, dass die Anzahl der Pilze während der ersten 14 Tage der Reifung signifikant abnimmt, bis zum Tag 90 aber wieder signifikant ansteigt. Ein Vergleich verschiedener Reifezeitpunkte zeigte, dass *Saccharomycetales* family *Incertae sedis* ASVs (Genus *Candida*, osmotolerante ascomycetes Hefen) während der ersten 14 Tage dominant ist, die relative Abundanz aber anschließend abnimmt. Im Gegensatz dazu war *Microascaceae* (genus *Scopulariopsis*) am Tag 0 und 14 nur in geringer Zahl vorhanden, nahm anschließend aber in beiden Einrichtungen zu. *Debaryomycetaceae* (Genus *Debaryomyces* und *Yamadazyma*) nahm nach dem ersten Tag der Produktion stark zu, anschließend aber während des gesamten Reifungsprozesses wieder ab.
Diese neuen Ergebnisse ermöglichen ein tieferes Verständnis des Reifungsprozesses von Vorarlberger Bergkäse, sodass die Verarbeitungs- und Reifeverhältnisse angepasst werden können und dadurch die Qualität des Produktes gesteigert werden kann.

4. Abbreviation list

A	Adenine
ASV	Amplicon sequence variant
BCE	Bacterial cell equivalent
BC	Before Christ
bp	Base-pair
C	Cytosine
°C	Degree Celsius
CO_2	Carbon dioxide
C_t	Threshold cycle
C_q	Quantification cycle
DADA	Divisive Amplicon Denoising Algorithm
dNTP	Desoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
FAM	Carboxyfluorescein
FCE	Fungal cell equivalent
Fig	Figure
G	Guanine
g	Gravitational force
g	Gram
HCl	Hydrogen chloride
gDNA	Genomic DNA
HTS	High Throughput Sequencing
H_2O	Hydrogen dioxide
IDF	International Dairy Federation
i.e	Id est (latin. that is)
IQR	Interquartile range
kB	Kilo Base
kg	Kilogram
LAB	Lactic acid bacteria

M	Marine agar
$MgCl_2$	Magnesium chloride
mm	Millimetre
mM	Millimole
min	Minutes
NaCl	Natrium chloride
ng	Nanogram
NH_3	Ammonia
nt	Nucleotide
nm	Nanometer
O_2	Oxygen
OTU	Operational taxonomic unit
PAB	Propionic acid bacteria
PCR	Polymerase Chain Reaction
PDO	Product of designated origin
pH	Potential of hydrogen
p-value	Probability value
Pmol	Picomol
qPCR	Real-time quantitative PCR
R	Programming language
sec	Seconds
SGA	Sabouraud Agar
T	Thymine
U	Unit
VB	Vorarlberger Bergkäse
YGC	Yeast Glucose Chloramphenicol Agar
YPD	Yeast extract Peptone Dextrose Agar
µl	Microlitre
µM	Micromole

5. Introduction

5.1. History of cheese production

Cheese is one of the most well-known food products in the world. It is not exactly known when the first milk or milk products were consumed by human, but it is commonly believed that a milestone occurred in the “Fertile Crescent” region [2].

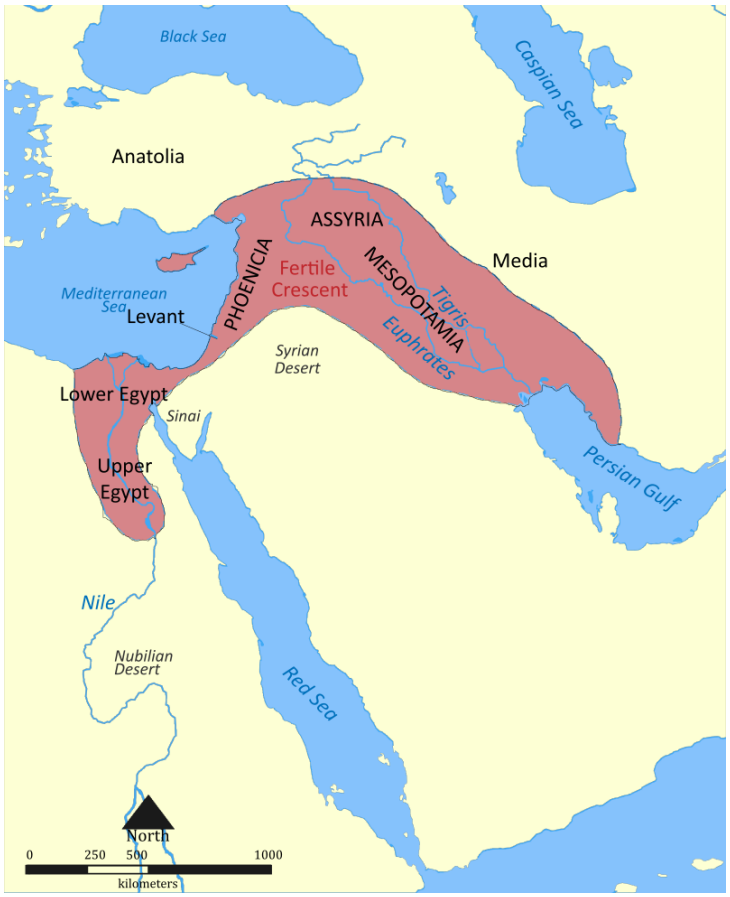

Figure 1. Neolithic development in the Fertile Crescent of southwest Asia lead to the domestication of diary animals. The area of the Fertile Crescent is highlighted in red [1]

Some 8000 years ago, the “Agricultural Revolution” took place in this region. The cultivation of field crops by sedentary Neolithic villagers likely attracted goats and sheep. The use of wool, milk and traction, so-called “secondary” products obtained from livestock without killing them, resembles an important step in the history of domestication during that time [3]. Soon humans recognized the nutritional value of milk produced by their domesticated animals. Some evidence of this process still exists, like shifts in the bone distribution of domesticated animals of that time and by the presence of milkfat residues in ancient pottery shards [4].

Before the development of pottery (~5000 BC), milk was usually stored in bags made of animal skins, in addition to the stomachs of slaughtered animals provided well-sealed containers for that use. The stomach tissue contains chymosin and pepsin, which leads

to the coagulation of milk during the storage phase. In the end, the coagulated curds were very different from to the ones produced by isoelectric acid precipitation. It was possible to convert the rennet-coagulated curds into a more stable product than acid curds. This way of coagulation became the predominant cheese manufacturing technique and was responsible for 75% of the world´s cheese production. People in ancient times were likely not aware of the microflora in raw milk and therefore had no knowledge of beneficial starter cultures. They simply took advantage of the microorganisms naturally present to coagulate the raw milk. For a long period of time, cheese making remained more art rather than a science until not long ago with the development of chemistry and microbiology. It has become possible to “manipulate” the process of cheese making in a more controlled fashion, but the microbial populations in cheeses still remain difficult to control due to their interactions and complex dynamics [5].

5.2. Diversity of cheeses, classification and principal categories of cheeses

Today there are 1400 cheese varieties listed by Jim Path from the University of Wisconsin (www.cdr.wisc.edu), and several attempts have been made to classify cheese varieties into meaningful groups. Several classification schemes rely on microbiological criteria, while others are entirely based on the description of the techniques used to produce the cheese [6].
Most classical classification schemes depend on exclusively one of the following criteria: milk type, coagulation method, cooking temperature of the milk, cheese composition, textural properties and characteristics of the ripening agent. There are just a few classification models that are based on a more integrative approach. Yet, this kind of grouping represents a more accurate picture of the cheese itself and the differentiation among the varieties.

Seen on an international scale, there are three major but different approaches; i) the “European” approach is mainly used in France, ii) the southern part of Europe uses the technological processes as the criteria of classification, while iii) the “Anglo-Saxon” classification model is mainly based on firmness that means the textural properties of cheese. The main goal of all classification systems is to provide a comprehensive and practical integrative classification model for a better understanding of the connection between the microbiological, technical and sensory characteristics of a cheese [7].

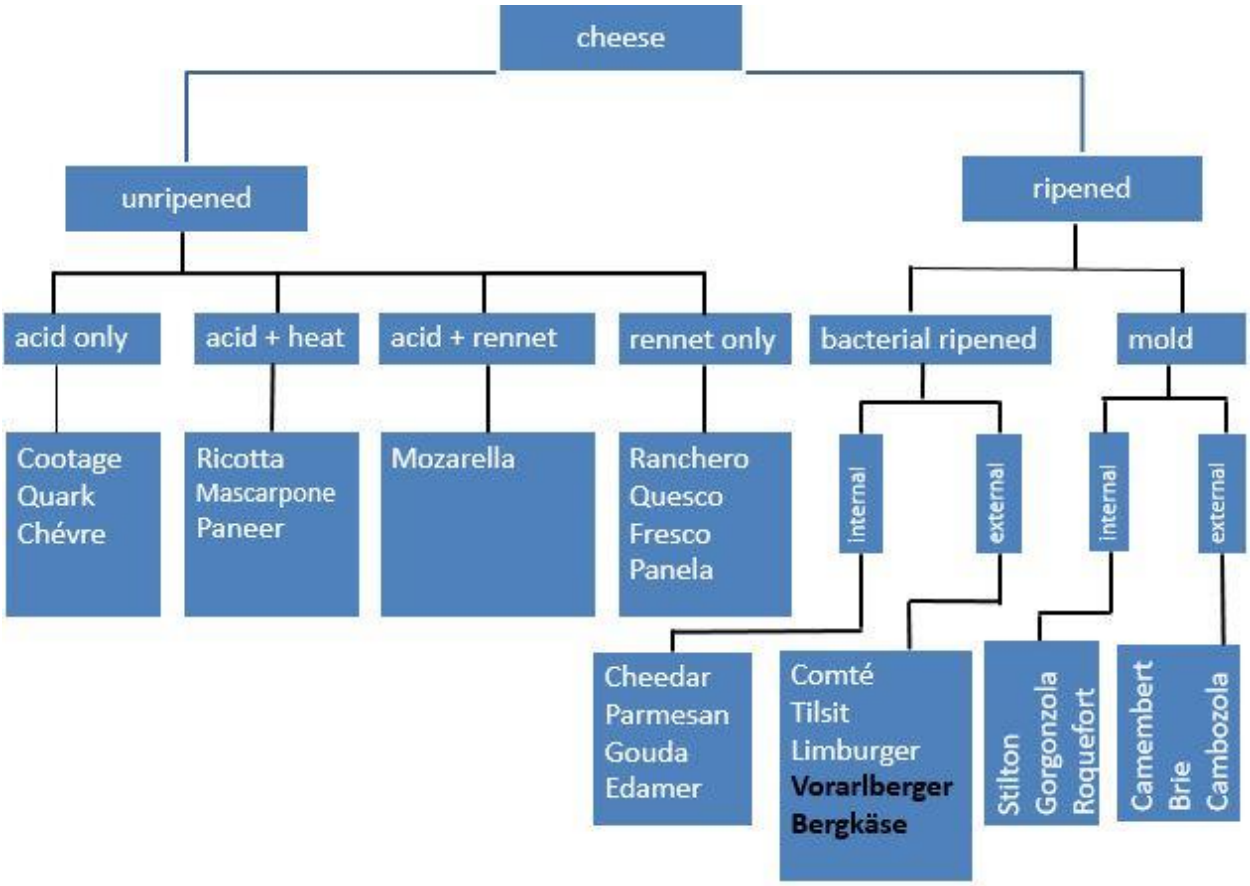

Figure 2. Flow chart expressing the different manufacturing methods resulting in the associated cheese variety. The Vorarlberger Bergkäse is highlighted in black.

5.3. Vorarlberger Bergkäse

The history of cheese making in the Alps goes back as early as the first century BCE. Its remote and mountainous regions made it necessary to perfect the technique of cheese making to produce food that is less perishable during the long winters. Additionally, the tillable land was very limited and thus it was farmed intensively. Fortunately, Alpine meadows provided ideal places for cows to graze, thereby providing milk for cheese manufacturing. Cheese making became a necessity for famers in these remote areas and small cooperatives emerged that produce cheese from an entire herd of cows [2].
Austrian Vorarlberger Bergkäse (VB), with its protected designation of origin (PDO), is an artisanal long-ripened hard cheese [8]. Vorarlberg, a federal state in the most western part of Austria, has a long tradition in raw milk hard cheese production. The regions Bregenzerwald and Walsertal in particular are still used today by small farms which herd up to 20 cows and produce around 4.450 tons of the traditional Vorarlberger Bergkäse per year using the long-established technologies. The local farmers from this area formed an

association called the “Käsestrasse Bregenzerwald” to promote local agriculture and products. Two companies dominate the cheese market in Vorarlberg nowadays: Rupp and Alma [9].
In the 21th century, traditional VB cheese is produced following a specific approach combining traditional methods and science in order to preserve the elements that contribute to the sensory characteristics of the cheese. Various factors are interacting to give a specific sensory characteristic and distinctiveness to the cheese.

5.3.1. Milk as the starting product

The VB production starts with the raw cow milk, which is gathered under strict quality guidelines for milk production in the area surrounding the cheese diary. Cattle feed must be natural and free of genetically modified organisms. The feeding of silage (fermented feed) is prohibited. This can lower the risk of introducing butyric acid producing bacteria in milk and cheese, which can result in late blowing [10]. Extensive grassland farming and no utilization of silage feeding are mandatories.
Raw milk has to be collected on a daily basis and be processed within 24 hours to reduce the populations of psychotropic bacteria in milk and later during the ripening process.

5.3.2. Addition of calves’ rennet, starter/whey cultures and curd cutting procedure

The non-pasteurized milk is partially skimmed leaving a fat content of around 3.3% and then coagulated using calf-rennet and a lactic acid culture (LAB).

5.3.3. Filling and pressing

Use of copper vats, instead of stainless steel, inhibits the formation of unpleasant taste due to the control of propionic acid bacteria and consequently propionic acid fermentation. In addition, the risk of gas overproduction is reduced. After the curd formation, heat is applied at approximately 51-52.5°C. Thereafter, the heated mass is pressed.

5.3.4. Brine salting and ripening

Formed cheese is soaked in brine with around 20% sodium chloride for 2-3 days and then ripened in cellars at a temperature of 12 to 15°C and a relative humidity between 90 and 95%. During ripening, the cheese wheels that weigh around 8 to 35 kg are regularly treated (brushed or rubbed) with brine twice a week to promote the formation of the typical taste and the ivory- to light yellow coloured rind. No external surface ripening cultures are applied during the ripening process, which makes the VB distinct from other similar products [8]. In some plants dry salting is applied at the beginning of ripening. The ripening time ranges from 3 to 18 months.

5.4. Microbiology on cheese rinds-bacteria and fungi

From the perspective of microbial ecology, the cheese core and cheese rind are fundamentally different ecosystems. Cheese compromises different pH values, salt concentration and oxygen levels coupled with large varieties of bacterial and yeasts communities within one product [11]. Due to the enzymatic activities occurring in the cheese rind, it has a major impact on the organoleptic properties of the cheese itself. Additionally the cheese rind itself provides a barrier against pathogens and spoilage microorganisms [12]. Raw milk cheeses are especially characterised by a high diversity in terms of different genera in the cheese rind. A similar diversity of microorganisms, the major contributors in the cheese making process itself, is not commonly found in pasteurized milk cheeses.

Artisanal raw milk cheeses like VB profit from microbial diversity as an enhancer of flavours and aromas. Due to the complexity and development of the microflora throughout the ripening process, it is difficult to focus on a single specific organism, or even the effects of possible interactions between different species. In addition, the conditions of the media, which changes throughout the ripening process, can facilitate the activity of dormant microorganisms that have remained inactive until a certain time point during ripening. All this phenomes account for the challenges in studying the complex ripening process of cheeses [13].

At the beginning of the ripening process, the microflora of the cheese rind is usually dominated by milk microflora and starter bacteria. The primary function of starter bacteria like *Lactococcus lactis*, a lactic acid bacterium added to the milk, is to break down lactose. Galactose is produced as a result of this break down, which is metabolized by *Debaryomyces*, the most frequent yeast at this stage of ripening [14]. This leads to deacidification of the cheese surface, an increase in pH is promoted, which facilitates the growth of salt-tolerant bacteria such as *Corynebacterium*, *Staphylococcus* and *Halomonas* [15, 16]. It is known that these microorganisms contribute to the cheese ripening since their enzymes are also involved in proteolysis, lipolysis and conversion of amino acids to certain flavour compounds [17].

Outside of the starter bacteria, long ripened cheeses house other bacteria. Therefore, they are called non-starter lactic bacteria (NSLAB) and are not deliberately added as part of the starter culture or as secondary adjunct cultures. Nevertheless, they act as advantageous contaminants. NSLAB are able to grow during the ripening but do not contribute to the production of acid but have furthermore an impact on flavour. The principal bacterial groups belonging to the NSLAB are *lactobacilli, leuconostocs, pedicocci* and *enterococci*. The main source for these bacteria is probably the milk, especially the raw milk. It is also known that the cheese environment contributes to the microflora of cheese. Studies have shown that the preposition to a factory specific flora impart distinctive flavour characteristics to the cheese produced in the given plant [18]. The flora is so complex that it is

inevitable that interactions between bacterial and fungal members of the inhabiting populations occur.
Generally, there are five biochemical activities mediated by NSLAB: citrate utilisation, proteolysis, amino acid catabolism, lipolysis and propionic acid fermentation.

- Citrate is present at low levels in the milk right from the production; it is also a precursor of diacetyl and acetate. Both are important flavour components and the CO_2 release is responsible for the eye formation and the whole cheese texture [19].
- Proteolysis is the ability of lactic acid bacteria (LAB) to meet their amino acid requirements from the hydrolysis of milk proteins. The peptides and amino acids released during that process act as precursors for the flavours that develop during the ripening in cheese [20].
- Amino acid catabolism can result in the formation of many flavour compounds that contribute to the final taste of the cheese [21]. The degradative mechanisms that occur during the cheese making process may include deamination, decarboxylation, desulphuration, oxidation and reduction reactions resulting in the formation of various amines, aldehydes, alcohols, indoles, carboxylic acids and sulphur-containing leftovers [22].
- Studies have confirmed the presence of lipase and esterase activities in cheeses. A main contributor to this is *Lactobacillus plantarum* with its lipase and esterase activity. Its abundance increases as the carbon chain length of the fatty acid decreases. The response of this activity to the variable salt, temperature and pH conditions is strain-dependent, but the perpetuation of these activities are vital for flavour formation [23].
- Propionic acid bacteria (PAB) contribute through propionic acid fermentation to a distinct flavour during ripening. The cheese appearance is also influenced through this metabolic pathway. They metabolise the lactic acid in the cheese curd:

  $3 \times \text{Lactate} \rightarrow 2 \text{ propionate} + \text{Acetate} + CO_2 + H_2O$

  Propionic acid bacteria are able to survive the relatively high cooking temperatures that occur in the early stages of cheese manufacturing. Interactions between PAB and other bacteria are important, PAB require proteolysis of casein by rennet and starter bacteria to stimulate their growth [24].

5.5. Mycobiome of cheese rinds

For many years, the role of fungi in cheese ripening was not well understood. Recent studies have helped to recognize their importance in terms of growth and chemical activities during the cheese-ripening process. Fungi are classified according to their morphologies and can occur in yeast, filamentous and dimorphic form. The filamentous fungi are also called molds and consist of a highly branched system of tubes called hypha and are multicellular, while yeast are typically spherical or cylindrical single cells. The dimorphic form can switch between the mold and yeast form. and can be therefore viewed as having either hyphal threads or as a single celled organisms under the microscope [25].

5.5.1. Filamentous fungi

Filamentous fungi are a type of fungus that grows in a multicellular form with filaments called hyphae. They contribute directly to the appearance of the cheese surface with their often-white mycelium, or in the case of *Penicillium roqueforti*, a variation of yellowish-green. *Penicillium camemberti* and *P. roqueforti* are also able to utilise lactic acid as a carbon source and therefore their growth leads to an increase in pH. Through their endopeptidase and exopeptidase activities, they make a major contribution to proteolysis in cheese and consequently soften the cheese. By lipolysis, the beta-oxidation of free fatty acids produces methyl ketones and their secondary alcohols. Both compounds contribute to the typical flavour of long-ripened cheeses. The increased pH level through filamentous fungi activity promotes the growth of bacteria. Additionally, produced tyramine, histamine and tryptamine through decarboxylation of amino acids are metabolised by coryneform bacteria [26].

5.5.2. Yeast

Yeast are eukaryotic single celled organisms, classified as members of the fungal kingdom. Approximately 1500 species are currently identified and distributed between the *Ascomycetes* and the *Basidiomycetes*. Some yeast are also used as culture additives in cheeses [27]. Bacterial surface-ripened cheeses rely on them, because of their ability to promote the growth of other advantageous microorganisms. Brie, Camembert or blue veined cheeses benefit from yeast. Yeast colonise aerobic cheese surfaces in particular during whey draining after moulding and before salting. Major sources of yeast in traditional cheeses are raw milk and various cheese processing equipment. The cheese plant environment, brine or the use of natural whey starters are also known as possible source of yeasts [18]. They are added to the milk itself and/or are used in the smear preparation. The most frequently occurring yeast species on surface-ripened cheeses are *Debaromyces hansenii* and *Geotrichum candidum*. Yeasts play a critical role in the deacidification and the formation of metabolites [2]. European cheeses are characterised by a complex surface smear that consists of yeasts and gram-positive bacteria. Other studies showed

that up to 30 different yeast species can be found on cheese rinds, while in the cheese core only lactose-fermenting yeast are expected [28].
Initially, established yeasts oxidise the available lactate to CO_2 and H_2O and release ammonia through deamination of amino acids. As mentioned above, this results in an increasing pH level on the cheese surface and a favourable state for the bacterial growth [29]. This interaction between the yeast and bacterial populations is essential for smear development and cheese ripening. Besides their importance in creating a hostable environment for bacteria, yeast are also capable of lipolysis through their lipase activity and are able to produce aroma compounds like ethanol, aldehydes and esters. For example, raw milk yeast isolates like *Candida* and *Kluyveromyces* have been reported as good lipase producers [15].

5.5.3. Methods to characterize microbial communities in cheese

To understand microbial processes during ripening, it is essential to monitor the complete flora. Ideally, the individual organisms should be accurately identified and characterised. Approaches used to achieve this, include culture-dependent and culture independent methods. Since the end of the 20th century, it has been possible to analyse microbial communities without the application of conventional culture-based analyses. Novel molecular methods emerged, including high-throughput sequencing (HTS), and therefore our view of microbial communities has changed rapidly. The combination of both culture-dependent and culture independent methods help to prevent the loss of less abundant species in the cultural dependent approaches [30].

5.5.4. Culture-dependent approach

Microbiota characterization using culture-dependent approaches starts with the selection of the media. Typical media contain a carbon source, water, various salts, a source of amino acids and nitrogen for growth. It must contain all essential nutrients required for microbial growth. Two major types of media are commonly used: a general media that is designed to generally sustain the growth of certain cultures and selective media that provides conditions favourable for a target organism. After successfully establishing a pure culture, next steps that can be made include determining the morphological traits of the culture, or simply by sequencing rRNA fragments to provide taxonomic information of the sample. However, all currently available culture-dependent techniques fail to reproduce the composition and diversity of environment samples found by using non culture-dependent techniques. This circumstance is mostly a consequence of the media composition that supports the growth a certain species that easily outnumbers others with more specific growth requirements [31].

5.5.5. Culture–independent approach

With the use of various PCR techniques, such as PCR-denaturing gradient gel electrophoresis, PCR-temperature gradient gel electrophoresis and PCR-single strand conformation polymorphism analysis, it is possible to carry out compositional analysis from various samples. Furthermore, several sequencing approaches are now available, including Sanger sequencing to obtain long reads (<500nt), useful for an accurate taxonomic analysis. State-of-the-art high-throughput sequencing (HTS) techniques like Illumina (Solexa), Ion Torrent (ThermoFischer) or Nanopore (Oxford) are used to generate massive amounts of data that help to describe an environmental sample in detail. Other approaches make use of clone libraries, typically used for whole genome sequencing projects. Recent advances in the development of software solutions facilitate the analysis of the gathered data and helps to define the microbial communities encountered in samples.

6. Aim of the thesis

The main focus of the thesis was to identify cheese-associated eukaryotes, mainly yeast and filamentous fungi, in the cheese rinds of Vorarlberger Bergkäse (VB). Culture-dependent (cultivation) and culture-independent (sequencing, quantification) approaches were used.

The specific aims were:

i) Monitoring of the differences in absolute abundance between two plants along the ripening process by using 18S rRNA gene TaqMan qPCR assay
ii) Cultivation of fungi
iii) Characterization of the fungi microbiome by using high throughput sequencing (Illumina Miseq)

7. Material and Methods

7.1. Cheese rinds samples

This thesis used the same samples for sequencing, qPCR and cultivation as described recently in detail in Schmitz-Esser *et al.* [31]. In brief, all samples were collected from two different ripening cheese production plants (A, B) located in Vorarlberg. With sterile scalpels, the entire surface of the cheese wheels was scraped resulting in 200 samples from five different ripening time points (Figure 3). The samples were collected directly after production (day 0), as well as after 14, 30, 90 and 160 days of ripening. For each time point, 20 cheese rind samples were used from different cheese wheels. Two grams of each sample were taken in duplicate and homogenized separately in 20mL sterile Ringer Solution (Braun, Austria) with a Stomacher 400 blender (Steward, London, UK). Samples were stored at 80°C at the Unit of Food Microbiology at the University for Veterinary Medicine (Austria, Vienna).

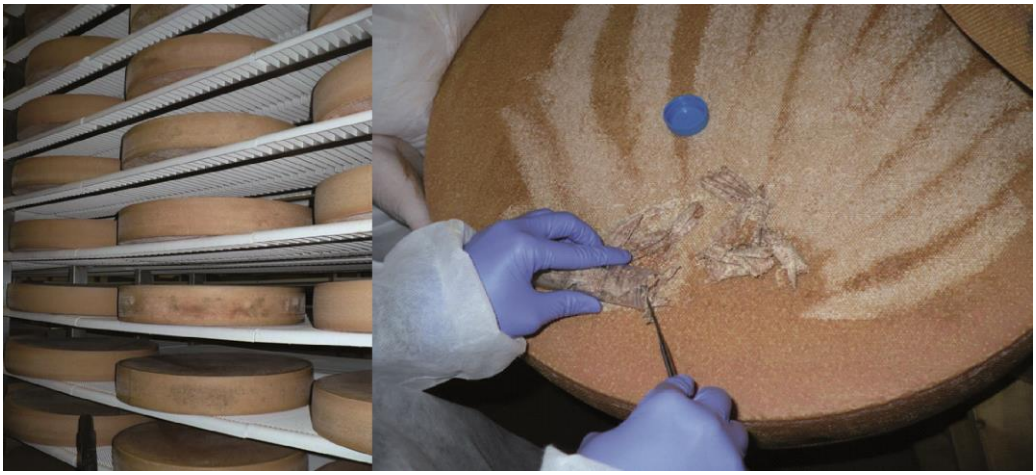

Figure 3. Sample collection in the ripening cellar © Monika Dzieciol

7.2. Cultivation media

A wide range of media is used for growing fungi. Preparation and selection of suitable media is one of the prerequisites to studying them. Moreover, different environments host a variety of fungi that grow with different requirements, including a specific level of salinity, pH, nutrient content and temperature. Hundreds of different cultivation media are available on the market and are optimized for a set of microorganisms. A key component is agar, a polysaccharide that adds no nutrients to a medium, but simply solidifies it. An acidic environment and an extent of carbohydrates are obligate for the optimal growth of most fungi. With the use of selective media, the growth of competing bacteria is inhibited or slowed down. The majority of fungi are difficult to isolate from their natural environments (e.g. infested soil or decaying organic material) due to the antagonistic and fast development of affiliated fungi, actinomycetes and bacteria that show higher growth rates. The isolation of many of these fungi can only be achieved by the use of selective media

e.g. with antibiotics. An antibiotic can either slow down the growth of unwanted organisms or inhibit it at all.

In this thesis, six different cultivation media were used to isolate yeast and filamentous fungi from the cheese rind samples. The selection of the media was based on the cloning results from Schornsteiner *et al*. [16].

1) Selective Agar for Pathogenic Fungi (P)
2) Yeast Glucose Chloramphenicole Agar (YGC)
3) Yeast Peptone Dextrose Agar (YPD)
4) Sabouraud Glucose Agar with Chloramphenicole (SGA)
5) Rose Bengal Chloramphenicole Agar (RBC)
6) Modified Marine Agar (M)

7.2.1. Selective Agar for Pathogenic Fungi (P)

P media contains cycloheximide for the cultivation of pathogenic fungi from heavily contaminated samples. Cycloheximide is produced by the bacterium *Streptomyces griseus* and acts as a eukaryote protein synthesis inhibitor. It blocks translational elongation and therefore inhibits the growth of rapidly growing contaminating molds [32]. Many pathogenic fungi are resistant to cycloheximide and it is speculated that its presence facilitates the transition from the parasitic to the saprophytic phase [33].

Agar-agar (Thermo Scientific, Austria)	12.5g/L
Soya peptone (Roth,Germany)	10g/L
Glucose (Merck, Germany)	10g/L
Cycloheximide (Roth, Germany)	0.4g/L

7.2.2. Yeast-Glucose-Chloramphenicol Agar (YGC) (Merck, Darmstadt, Germany)

YGC medium is recommended for the enumeration of yeast and mould in dairy products by the International dairy Federation (IDF). The growth of bacteria is decreased by the use of 0.05g/L chloramphenicol, while the high concentration of glucose (30g/L) favours the growth of yeast and filamentous fungi.

Agar-agar	15g/L
Yeast extract	5g/L
Glucose	30g/L
Casein peptone	5g/L
Chloramphenicole	0.05g/L

7.2.3. **Yeast Extract Peptone Dextrose Agar (YPD)**

YPD is a complete medium for the growth of yeast cultures. Yeast are unicellular eukaryotes and chemo-organotrophs that utilize organic compounds as a source of energy. Glucose serves as the carbon source while peptone acts as source of nitrogen, vitamins and minerals.

Agar-agar (Thermo Scientific)	15g/L
Glucose (Merck)	15g/L
Casein Peptone (Roth, Germany)	20g/L
Yeast Extract (Biokar, France)	10g/L

7.2.4. **Sabouraud Glucose Agar with Chloramphenicol (SGA) (SIGMA-ALDRICH, Vienna, Austria)**

SGA is a growth medium containing peptones to cultivate dermatophytes and other types of fungi. It was developed in 1892 by Raymond Sabouraud and named after him. Chloramphenicol is used against the growth of unwanted microorganisms. It is a broad-spectrum antibiotic that inhibits protein synthesis by stopping the protein chain elongation by binding to A2451 and A2452 residues of the 23S rRNA preventing peptide bond formation [34]. Additionally, the acidic pH of 5.6 in traditional Sabouraud Agar inhibits bacterial growth.

Agar-agar	15g/L
Meat peptone	5g/L
Casein peptone	5g/L
Dextrose	40g/L
Chloramphenicole	0.05g/L

7.2.5. **Rose-Bengal Chloramphenicol Agar (RBC) (Merck, Darmstadt, Germany)**

Rose Bengal Chloramphenicol (RBC) Agar is a selective medium especially created for the enumeration of moulds and yeasts in foods. The main component of rose Bengal (4,5,6,7-tetrachloro-2´,4´,5´,7´-tetraoidofluorescein), is a stain commonly used in the preparation of *Foraminifera*. In cultivation media, it not only restricts the size and height of mould but assists in enumeration due to the uptake of the fungi colour [34]. The growth of bacteria is inhibited by the selective agent of chloramphenicol.

Agar-agar	15.5g/L
Mycological peptone	5g/L
Glucose	10g/L
Potassium dihydrogen phosphate	1g/L
Magnesium sulphate	0.5g/L
Bengal rot	0.05g/L
Chloramphenicole	0.1g/L

7.2.6. **Modified Marine (MM) Agar (Carl Roth, Karlsruhe, Germany)**

Marine broth is mostly used for cultivating heterotrophic marine bacteria. In this study a modified version is used to attract halophilic microorganisms inhabiting the cheese surface. The NaCl content and various supplements simulate the salty environment after production with the cheese wheel and the numerous resulting events during ripening. Preparation was conducted using 40.1g/L Marine broth (Carl Roth) and then adding further supplements listed below.

Content of Marine broth:

Sodium chloride	19.4g/L
Magenisum chloride	8.8g/L
Peptone	5g/L
Sodium sulphate	3.24g/L
Calcium chloride	1.8g/L
Yeast extract	10g/L
Potassium chloride	3.24g/L
Sodium bicarbonate	1.8g/L
Ferric citrate	0.1g/L
Potassium bromide	0.08g/L
Strontium chloride	0.034g/L
Boric acid	0.022g/L
Sodium silicate	0.004g/L
Sodium fluoride	0.0024g/L
Ammonium nitrate	0.0016g/L
Disodium phosphate	0.0008g/L

Further supplements:

Yeast extract (Biokar)	10g/L
Agar-agar (Thermo Scientific)	15g/L
Chloramphenicole (Applichem, Germany)	0.05g/L
Glucose (Merck)	20g/L
Soya peptone (Carl Roth)	5g/L
Casein peptone (Carl Roth)	5g/L

The pH of all media were adjusted using 37% hydrochloric acid (HCl) and sodium hydroxide (NaOH) pellets. All plates were stored aerobically at 30°C except for RBC these plates were stored at 25°C.

7.3. Sample preparation for cultivation approaches

One g cheese rind was resuspended in 10ml sterile Ringer solution (Braun). After one min in double stomacher bags at the highest setting in a STOMACHER 400 (Seward, West Sussex, United Kingdom), the sample was diluted. Six tenfold dilution steps were carried out. Briefly, 100 µl of undiluted sample was resuspended in 900 µl Ringer Solution (Braun). 100 µl of each dilution step was then plated using a modified sterile Pasteur pipette 230mm (Assistant, Germany) on six selected culture media. All plates were incubated upside down to prevent the formation of condensation on the agar. Additionally, an airtight plastic bag was used to prevent dehydration of the medium.

7.3.1. Recultivation

Pure isolates with different morphologies were randomly picked and re-cultivated on agar plates of the same type (Figure 4 and 5). To prevent a cross-contamination, filamentous fungi and yeasts were cultivated separately.

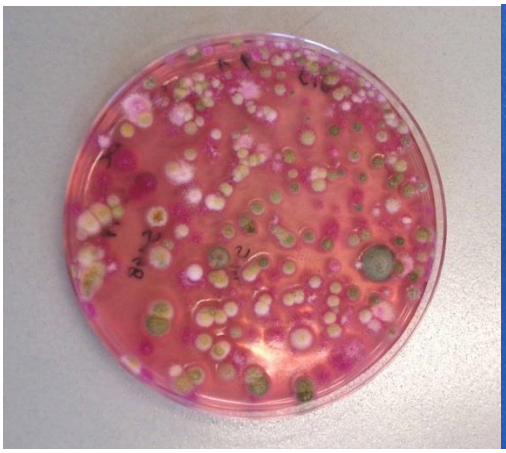

Figure 4. RBC plate of the highest dilution after incubation at 25°C

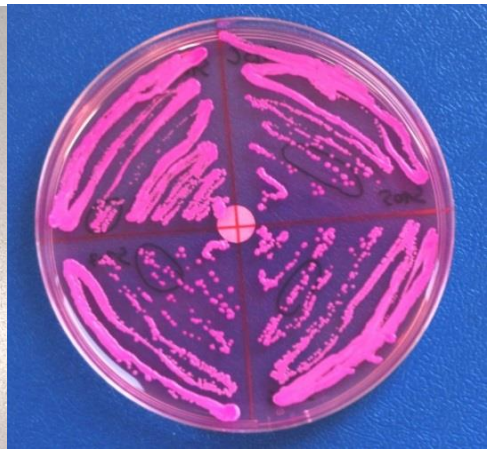

Figure 5. Four randomly picked colonies streaked out on the RBC plate.

7.4. DNA extraction

Fungi differ greatly in the presence and composition of their cell walls or capsules; thus, they are not readily susceptible to lysis. Similarly, the biochemical and physical properties of their habitat vary, especially in the content of saccharides that interfere with many standard protocols. Therefore, there is no universal DNA extraction method that will work well for all fungi and environments, especially in the cheese rind matrix. To overcome these challenges, different extraction protocols were tested (these included 5% Chelex®-100 [35, 36], autoclaved silica-based sand [37], beat beating tube, lysozyme [88] and commercial extraction kits DNeasy PowerSoil (Qiagen, Germany); NucleoSpin tissue (Macherey-Nagel, Austria)) in order to yield sufficient gDNA quality and purity of all samples.

Adding the chelating Chelex® 100 solution from Bio-Rad (California, USA) to the samples binds the Mg^{2+} cations, which are essential cofactors for DNases that would degrade the free DNA after successful cell lysis. DNases would render the DNA unsuitable for PCR and the downstream analysis. Random cheese rind samples were tested using four extraction methods followed by the PCR confirmation and gel-electrophoresis (Figure 6) to achieve the best approach.

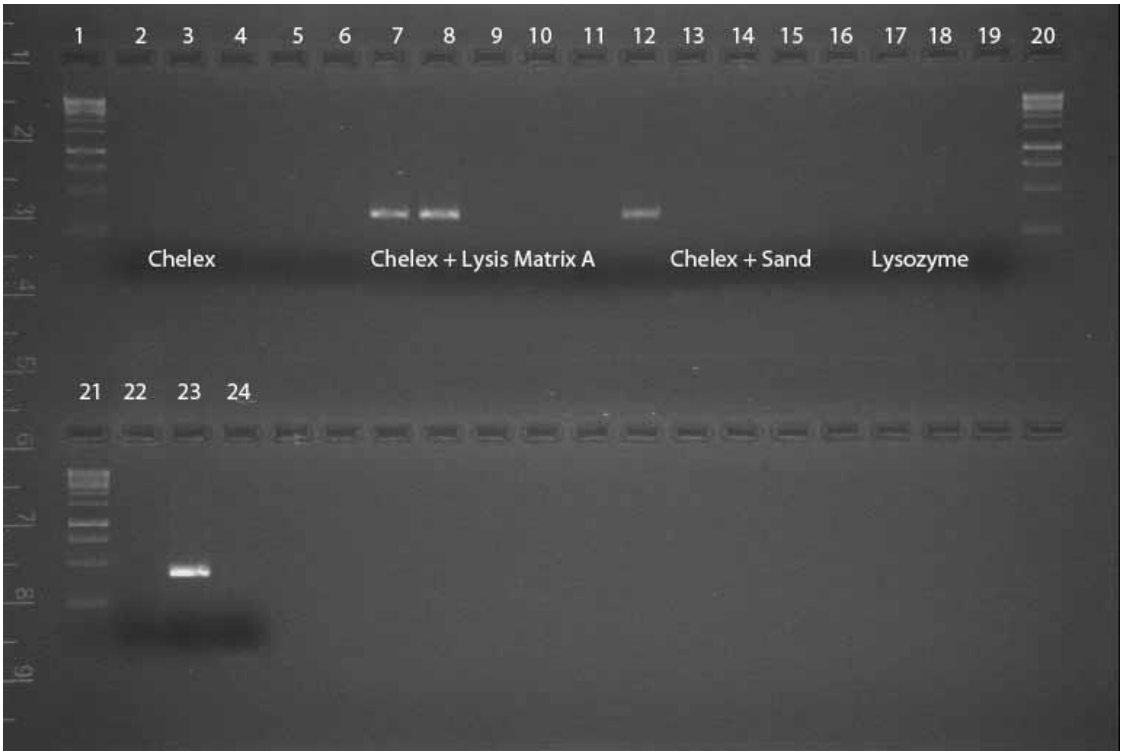

Figure 6. 1.5% electrophoresis gel of PCR products of 18S rRNA gene. Two of four samples (lane 7 and 8) were positive when using the Chelex 100® and "Lysis Matrix A" approach. Marker: 1kb DNA ladders (lane: 1, 20 and 21) were used to verify the size of the fragments. Lane 23: positive control (W303 isolate).

7.4.1. Basic principle of polymerase chain reaction (PCR)

To amplify the genes necessary for identification, it is required to run a polymerase chain reaction (PCR). By using small amounts of DNA sequences, it is possible to generate thousands to million copies of a particular DNA segment between 0.1 and 10 kb in length. The amount of the final product is limited by the available deoxynucleoside triphosphates (dNTPs). A basic PCR set-up contains several components including a heat resistance DNA polymerase, an enzyme that polymerizes the DNA strands. The heat resistance is due to the high temperatures during the denaturation phase. There are further a set of two DNA primers that are complimentary to each end of the DNA target of the sense and anti-sense strands that provide a binding site for the polymerase. Additionally, there are the dNTPs, the building blocks of the newly synthesized strands, and a buffer solution providing a proper environment for optimal amplification and stability of the DNA polymerase. A thermal cycler is used for heating and cooling the reaction that is carried out in a small reaction tubes according to a specific thermal profile [38].
In this thesis, for the taxonomic identification of the samples, the 18S rRNA gene and the ITS2 region was used. The slow evolutionary rate and the exposure to similar selective forces make the 18S rRNA gene usable for the identification of even closely related eukaryotic organisms. Sequence comparison of the ITS region is widely used in taxonomy because of its favourable properties, such as the small size, high degree of variation and highly conserved flanking sequences. The ITS2 region lies between the 5.8S rRNA gene and the 26S or 28S rRNA gene (Fig. 7) [39].

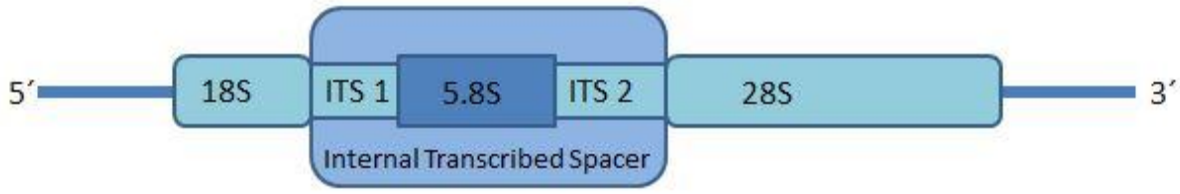

Figure 7. Schematic representation of eukaryotic rRNA operons. Positions and orientation is schematically indicated. Internal transcribed spacer 2 (ITS2) is situated between 5.8S large-subunit ribosomal (rRNA) and 28S large-subunit rRNA genes in the chromosome.

In this thesis two PCRs were used for amplification of i) 18S rRNA gene and ii) the ITS2 region: i) The 18S rRNA gene (864 bp) PCR was performed using the EK528F (5'- CGGTAATTCCAGCTCC-3' [40] and U1391R 5'-GGGCGGTGTGTACAARGR-3' primers [41]. Each reaction was performed in a PCR tube with a final volume of 25µl, containing DEPC-treated water (Thermo Scientific, Austria), 0.2pmol/µl of each primer, 200 µM of dNTP´s Mix (Thermo Scientific, Austria), 2 mM $MgCl_2$ (Invitrogen, Carlsbad), 2mM 10 × PCR puffer (Invitrogen), 0.625 U *Taq* Platinum polymerase ® (Invitrogen, Carlsbad) and 1 µl of template DNA. 18S rRNA gene PCR was performed under the following conditions: an initial denaturation at 95°C for 5 minutes, 35 cycles at 94°C for 40 seconds, annealing at

56°C for 40 seconds, elongation at 72°C for 1 minute and a final elongation phase of 7 minutes at 72°C.
ii) To amplify the ITS2 region, a primer combination of ITS3 forward [42] and ITS4 reverse [42] was used. The final reaction of 25µl consists of DEPC-treated water, 0.2 pmol/µl of each primer, 200µM dNTP´s (Thermo Scientific), 200nm $MgCl_2$ (Invitrogen), 10×PCR puffer solution and 0.625 U *Taq* Platinum polymerase ® (Invitrogen). Following thermal profile was used: initial denaturation at 95°C for 5 minutes followed by 35 cycles of 94°C for 40 seconds annealing at 56°C for 40 seconds and elongation at 72°C for 1 minute and a final elongation at 72°C for 7 minutes. PCRs were carried out in the Doppio Thermocycler (VWR, Vienna, Austria).

7.4.2. Basic principle of Gel-electrophoresis

Gel electrophoresis is a method used to separate macromolecules like DNA, RNA and proteins based on their size and charge. A matrix of agarose separates the larger molecules from the smaller when an electric field is applied and the negatively charged nucleic acid molecules migrate through the pores. This phenomenon is called sieving [43]. With a stain such as PeqGreen® (VWR, Vienna, Austria), the accumulation of DNA fragments becomes visible under ultraviolet light.
In this thesis, all PCR products were run on a 1.5% TBE agarose gel for 30 minutes at 120V (BioRad Power Pac 1000). The PCR products were visualized under UV light using the BioRad GelDoc 2000 (Bio-Rad laboratories, Vienna, Austria).

7.4.3. Sanger-Sequencing

Sanger sequencing of the obtained fragments was performed by LGC Genomics (Germany, Berlin).

7.4.4. Quality control

After receiving the sequence data from LGC, a fast quality control was performed by checking the resulting chromatograms. To visualize the .ab1 files, FinchTV (version 1.4.0) was used on a Microsoft Desktop machine.

7.4.5. Blastn/Unite

Taxonomic classifications (18S rRNA gene, ITS2 region) were determined by comparing sequences to the closest related type strains in the NCBI [44] and the UNITE databases [45].

All obtained sequences were confirmed using the NCBI nucleotide database with BLAST® (https://blast.ncbi.nlm.nih.gov). The blast algorithm is used to compare amino-acid sequences of proteins or the nucleotides of DNA/RNA sequences. When it is used to identify a specific sequence it will identify library sequences that resembles the query sequence to a certain amount.

For the identification of the cheese rind samples, the database used was not restricted to type material to enhance the chance of finding a matching sequence. The BLAST database used included ITS regions and 18S rRNA gene sequences. In addition, UNITE (https://unite.ut.ee/) was used for redundancy as it contains a database with 536.881 fungal sequences.

7.5. Methods qPCR

7.5.1. Basic principle of the real-time quantitative PCR (qPCR)

A very accurate technique to determine the concentration of certain DNA molecules within a sample is the quantitative real-time polymerase chain reaction (qPCR). The method monitors the amplification process of a target DNA molecule during the PCR. Two widely applied methods for the real-time detection of PCR products are: i) usage of non-specific fluorescent dyes that intercalate with the minor groove and ii) a sequence specific DNA probe. This specific probe carries a fluorescent reporter which only enables detection after hybridization of the complimentary sequence with the target.

i) This method uses a DNA binding dye that binds unspecifically to all double-stranded (ds) DNA in the PCR reaction, emitting fluorescence from the dye. With every cycle, the concentration of DNA products increases. This leads to an increase in fluorescence measured at each cycle. Double strand DNA dyes like SYBR Green® (Thermofisher, Waltham, USA) will bind to all dsDNA´s, including nonspecific PCR products. When primers are attached to each other a primer dimer forms, the unspecific DNA dye will intercalate with it leading to potentially inaccurate results.

ii) The second method requires a fluorescent reporter probe (e.g. TaqMan) that contains the complimentary sequence to the target DNA fragment. Therefore, the use of a reporter probe significantly increases specificity. Additionally it enables performing this technique even in the presence of other dsDNA´s. Due to the high specificity of fluorescent reporter probes interferences caused by primer dimers, which are still possible by-products in PCR are prevented. This method relies on a quencher of fluorescence at one end and a florescent reporter on the opposite. The close proximity of them inhibits the detection of any fluorescence. The complimentary sequence lies in between and is necessary for the specificity of this method it binds to the target DNA fragment.

Taq polymerase is an enzyme derived from a thermophilic bacterium, *Thermus aquaticus*. It was first isolated in 1976 and is able to break the probe down with its 5´ to 3´ exonuclease activity [46]. The quencher can no longer inhibit the fluorescence and a signal is detectable after excitation with a laser. The breakdown of the probe followed by the release of the reporters leads to an increase in detectable fluorescence indicating the proportional amount of target present.

The amplification of non-specific PCR products must be avoided for exact quantification a melting curve analysis helps to identify primer dimers and rule out a non-specific PCR. The melting process of double-stranded DNA induces a sharp reduction of fluorescence signal around the melting temperature of the target PCR product, resulting in a clear peak. Therefore, different fragments with unequal length and base composition have variable melting temperatures and appear as separate peaks.

7.5.2. 18S rRNA gene qPCR assay

In order to amplify the total fungal community of the VB cheese rinds, a TaqMan qPCR assay based on a conserved region of the 18S rRNA gene (351bp) was used [47]. The specificity of the used primer pair (forward primer 5´-GGRAAACTCACCAGGTCCAG-3´; reverse primer 5´-GSWCTATCCCCAKCACGA-3´) has been supported by other studies [48, 49] and therefore has been recommended for fungal quantification. To address a wide variety of fungi, the primers are degenerated, meaning that single bases are substituted with a population of primers with similar sequences that cover all possible nucleotide combinations for a given protein sequence [50]. For the forward primer, it is the third position, marked with an "R" that stands for pu**R**ine (Adenine or Guanine) according to the IUPAC (International Union of Pure and Applied Chemistry) nucleotide code. In the reverse primer, three positions are substituted that are marked with "S" ("**S**trong" base pairing, G or C), "W" ("**W**eak" base pairing) for A or T and "K" (**K**eto group on base) for G or T [51].
To increase the probe binding affinity to the target sequence, the FungiQuant Probe (FungiQuant-Prb (FAM) 5´ FAM-TGGTGCATGGCCGTT-3´-MGBEQ), is a non-degenerated TaqMan minor-groove binding probe, consisting of a fluorophore (FAM, 6-carboxyfluorescein) that is attached to the probe. The fluorophore is a single isomer derivative of fluorescein amidite (FAM) and has an absorbance maximum at a wavelength of 495nm and an emission output of 520nm. It is compatible with most fluorescence detection equipment. The nucleic sequence of the probe is complimentary to a certain region of the PCR product.

7.5.2.1. Reproducibility- DNA standard curves and copy numbers

DNA standard curves used for the 18S rRNA assay were generated by plotting the quantification cycle (C_q) values of the target, against the log initial quantities of the tenfold dilutions (Figure 8). The cycle threshold (C_q) value of a reaction is defined as the cycle number when the fluorescence is above the background signal and indicates when the fluorescence signal enters the exponential phase. This exponential phase is when the PCR product doubles with every cycle conducted.
The DNA standard for the 18S rRNA qPCR was prepared using the strain *Saccharomyces cerevisiae* NCPF 3178 (National Collection of Pathogenic Fungi's, United Kingdom) and had an A260/A280 ratio of 1.7, indicating optimal DNA purity. The strain was cultivated on the Yeast Extract Glucose Chloramphenicol Agar (Merck KGaA, Darmstadt, Germany) at 37°C, for 48 hours at aerobic conditions.

For the DNA extraction, a loop of yeast culture was resuspended in 1 ml Ringer Solution (Braun) and centrifuged for 5 min at 8,000 × g. The pellet was processed with the NucleoSpin ® Tissue kit (Macherey-Nagel GmbH&Co.KG, Düren, Germany).
DNA concentration was determined by using a Qubit® 2.0 Fluorometer (BroadRange Assay Kit; Thermo Fisher Scientific, Vienna, Austria). An equation listed below was used to calculate the copy numbers of the standards [52].

$$\text{DNA copies} = \frac{(6.02 \times 1023) \times \left(\frac{copy}{mol}\right) \times \text{DNA amount (g)}}{\text{DNA legnth (bp)} \times 660 \left(\frac{\frac{\text{g}}{\text{mol}}}{\text{bp}}\right)}$$

The 18S rRNA gene target with the length of 351 bp has a weight of 228,150 dalton (molecular mass unit equivalent to the 12th of a single carbon isotope ^{12}C) or equivalent of 3.79×10^{-22} in kg or 2.64×10^{9} per ng. Five µl of the DNA template was used in the qPCR, thus the weight was multiplied by 5 (1.32×10^{10}). The 18S rRNA operon copy number occurs in tandem repeats and range from 60 in the basidiomycete *Coprinus* to 220 in the ascomycete *Neurospora* [53]. Indeed, a common filamentous fungi *Aspergillus fumigatus* has between 38 and 91 copies per genome. Due to the fact, that the number of operon copies of rRNA is highly variable in fungal genomes (even within the same species), one cannot determine an average number for that applies for general environmental fungi [54].
About 150 copies per haploid genome in yeast *Saccharomyces cerevisiae* are produced therefore, we converted our fungal DNA and defined *Saccharomyces cerevisiae* as fungal cell equivalent (FCE) in this study. For these 150 operons, copy number was taken into account when extrapolating fungal cell equivalents from qPCR. Copy numbers (1.32×10^{10}) were divided through 150 that results in 8.8×10^{7} FCE copies (1ng/µl) per 5µl DNA or per 0.5 g cheese rind.
The DNA of the strain was adjusted to a concentration of 0.1 ng/µl in DEPC-treated water (8.8×10^{6} FCE/5 µl) and applied for the qPCR.

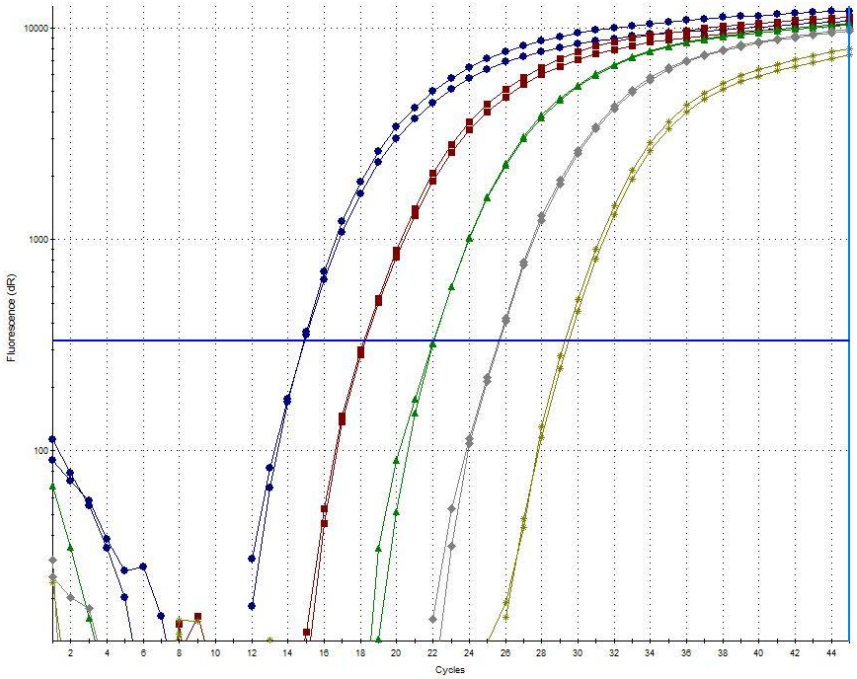

Figure 8. Amplification plots of qPCR. Amplification plots based on C_q values obtained by TaqMan 18S rRNA assay detected in the FAM channel. A 10-fold dilution series of Saccharomyces cerevisiae NCPF 3178 purified genomic DNA were used as a template to generate standard curves of the assay. Standard curves were generated by plotting the log of initial quantities of 10-fold dilution series genomic DNA against the obtained C_q value from the assay (range from 8.8×10^6 (0.1 ng/µ) to 8.8×10^2 FCE/5 µl).

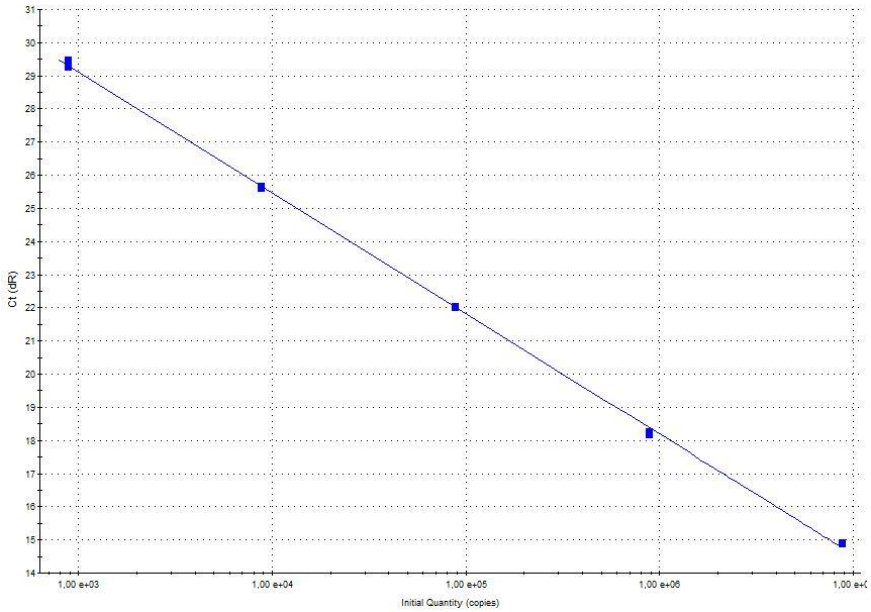

Figure 9. 18S rRNA gene qPCR standard curve. The efficiency of the qPCR assay was 92.5% with an R^2 of 0.999.

7.5.3. Quantitative real-time PCR conditions

Single amplification reaction for 18S rRNA gene qPCR consisted of 10.45 µl diethylpyrocarbonate (DEPC)-treated water, 2.5µl 10×buffer (Invitrogen, Carlsbad, USA), 1.75 µl 3.5mM $MgCl_2$ (Invitrogen), 1.5µl 300nM of each primer, 1.5 µl of the probe, 1 µl 200 µM of each dNTP (Thermo Fischer Scientific), 0.3 µl 1.5 U of Platinum Taq DNA polymerase (Thermo Fisher Scientific) and 5 µl template (genomic DNA). The quantification of DNA was performed using a Mx3000P qPCR instrument (Strategene, La Jolla Ca USA) (Software v 4.10) after initial denaturation at 94°C for two min, followed by 45 cycles of 94°C for 30 s, 60°C for one min. Negative controls without template were included in each qPCR reaction. Each qPCR reaction was run in duplicate with a final volume of 25µl (20µl MasterMix and 5µl template), using MicroAmp 0.2 ml optical tubes sealed with MicroAmp optical 8-cap strips (Applied Biosystems, Foster City, CA, USA).
The mastermix was prepared in a laminar flow (BSB 4 A, GELAIRE Flow Laboratories, Australia) previously sterilized by UV light. All qPCR components and DNA were kept on ice, and stored at -20°C.

7.5.4. Statistical Analysis

The qPCR data (FCE per 0.5 g cheese rind) were analyzed using R (version 3.2.5, psych package 1.6.12). The dataset was divided into 10 different subsets based on the location (A, B), target (18S rRNA) and days of ripening (0, 14, 30, 90 and 160). Because the Shapiro-Wilk test showed normal distribution only for 3 of the 10 subsets, all subsets were described by median and interquartile ranges (IQR). Furthermore, the Wilcoxon Signed-Rank test was used to determine statistical differences between subsets with the same location and same target based on days of ripening. Briefly, Wilcoxon signed rank test compares the sampling days with each other to assess whether their mean ranks differ and represents the outcome as a p-value. It highlights the abundance differences that are statistically significant. The Kendall rank correlation coefficient was used to measure the ordinal association between both ripening plants. To describe the relationship between both data sets, it uses values between -1 and 1. A completely independent dataset would generate a coefficient of approximately zero.

7.6. Illumina

In order to investigate microbial composition in VB rinds throughout time, the DNA purified from the 200 samples described above, that were taken from two VB producing plants (A and B) at different ripening points, were pooled regarding their ripening time points (0, 14, 30, 90 and 160 days) and submitted to further gene-targeted amplicon sequencing.
Fungal diversity was studied by amplification of the internal transcribed spacer 2 (ITS2) region (*ca.* 300-400 bp), with the primers set ITS3-5'-GCATCGATGAAGAACGCAGC-3' and ITS4-5'-TCCTCCGCTTATTGATATGC-3' [42] and further sequencing by using a

MiSeq Illumina platform, leading to 2,334,863, 250 bp paired-end reads. PCR amplification, sample multiplexing, library preparation and sequencing were performed by Microsynth AG (Balgach, Switzerland).
Afterwards a sequence analysis was performed using the DADA2 (version 1.12) plugin with R (version 3.2.5).

7.6.1. Basic principle of Illumina sequencing

The Illumina sequencing technique is founded on reversible dye-terminators that enable the identification of single bases as they are introduced one by one into the DNA strands. This process takes place inside of a glass flow cell that has its bottom coated with oligonucleotides. The purified DNA gets sliced into several much smaller pieces and adapters are added to the ends. The so modified DNA is then loaded onto the chip. In this place amplification and sequencing occurs. The oligonucleotides act as anchors for the modified DNA and grab the complementary sequences. When the fragments are finally attached, cluster generation begins. In this way thousand copies of each fragment ca be created at the same time. In the next step, primers and modified nucleotides are added onto the chip. These specialised nucleotides carry reversible 3´blockers that force the polymerase to add only one nucleotide at a time additionally to a fluorescent tag. After each round of synthesis, a picture is acquired by a camera. Through the wavelengths emitted by the fluorescent tag, a computer can determine what base was added and registers it for every spot on the chip. Non-incorporated molecules are simply washed away after each round and a chemical deblocking step is used to remove the 3´terminal blocking group and the dye.

7.6.2. Data overview

Microsynth (Switzerland) generated a total of 20 files with an average read length of 250bp. A total of 2,334,868 reads with 582,661,505 bases are used for further analysis. Detailed information for each sample is listed below in Table 1.

7.6.3. Data analysis

Data analysis was performed in DADA2 (version 1.12). DADA2 offers a unique error-correction method disentangling biological variation from amplicon sequencing errors designed to be applicable to the Illumina sequencing method. Indeed, DADA stands for Divisive Amplicon Denoising Algorithm, a model-based approach for correcting amplicon errors without constructing operational taxonomic units (OTUs) that are clusters of sequences with a fixed dissimilarity threshold of usually 3%. Additionally, the DADA2 workflow implements filtering, dereplication, sample interference, chimera identification and merging of paired-end reads [56].

Sample	Reads	Bases	Mean Read length	Mean Quality
A-0_S163_R1_001	122355	30526299	250	37
A-0_S163_R2_001	122355	30500437	249	36
A-14_S175_R1_001	99450	24707076	249	37
A-14_S175_R2_001	99450	24705280	249	36
A-30_S187_R1_001	127937	31930573	250	36
A-30_S187_R2_001	127937	31928085	250	34
A-90_S104_R1_001	83667	20905546	250	36
A-90_S104_R2_001	83667	20865197	249	34
A-160_S116_R1_001	122238	30531712	250	35
A-160_S116_R2_001	122238	30517138	250	34
B-0_S103_R1_001	151527	37864427	250	37
B-0_S103_R2_001	151527	37870526	250	36
B-14_S115_R1_001	100484	25110468	250	37
B-14_S115_R2_001	100484	25108728	250	36
B-30_S127_R1_001	120024	29962425	250	35
B-30_S127_R2_001	120024	29970853	250	34
B-90_S139_R1_001	99005	24705798	250	36
B-90_S139_R2_001	99005	24698610	249	33
B-160_S151_R1_001	140747	35124528	250	36
B-160_S151_R2_001	140747	35127799	250	34

Table 1. Summary of ITS sequencing output generated by Microsynth AG.

7.6.3.1. DADA2 pipeline

The regularly occurring errors in Illumina- data was addressed by using the newest version of the DADA algorithm. Therefore, the DADA2 plugin (version 1.11.1) for R was applied. Prior to any analysis, the sequences were inspected and the quality from the forward and reverse reads was visualized.

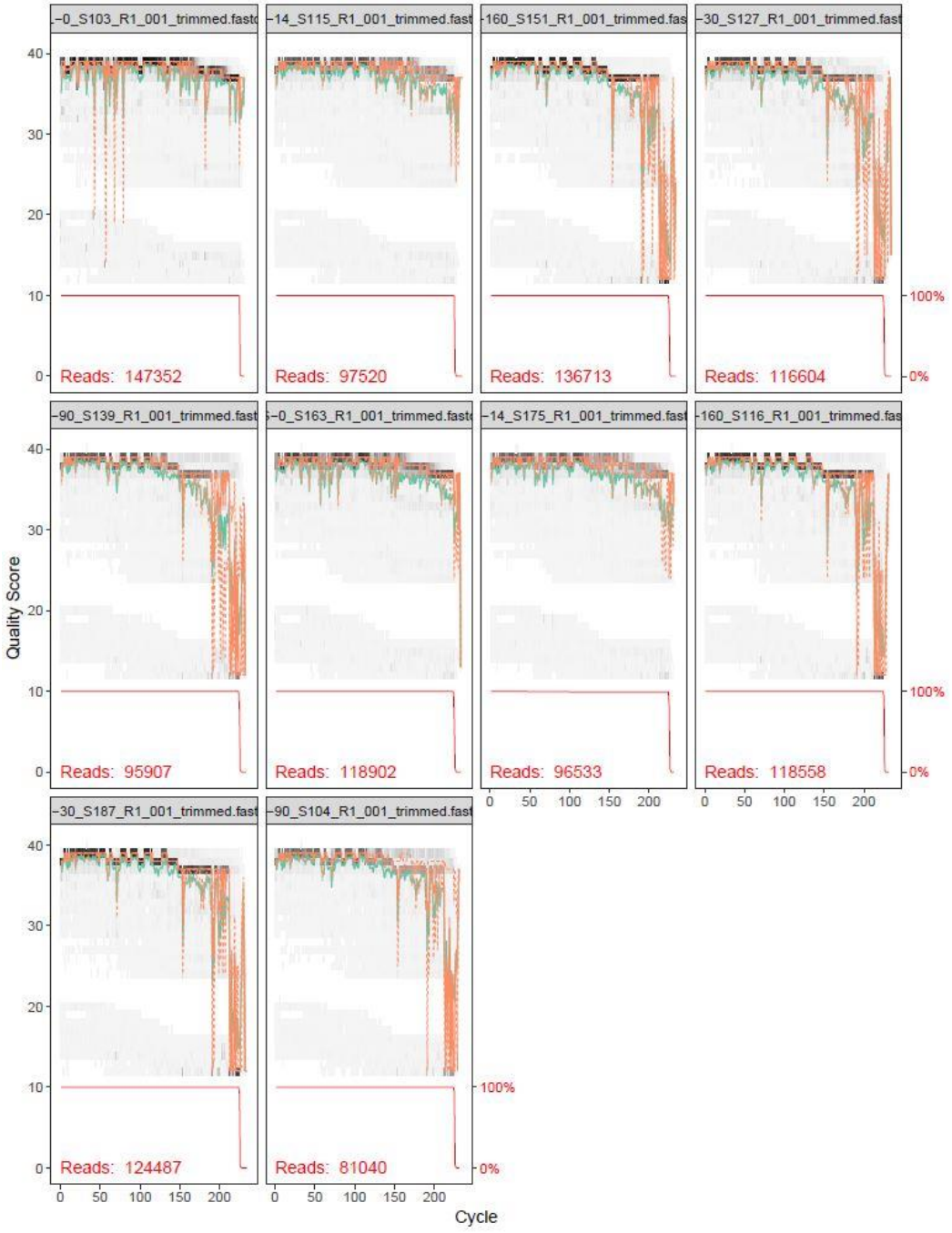

Figure 10. Quality plots of the forward reads. Generated quality plots by the DADA2 plugin. The green line shows the median quality score, and the quartiles of the quality score distribution by the orange lines. The red line indicates the scaled proportion of reads that extend to at least that exact position.

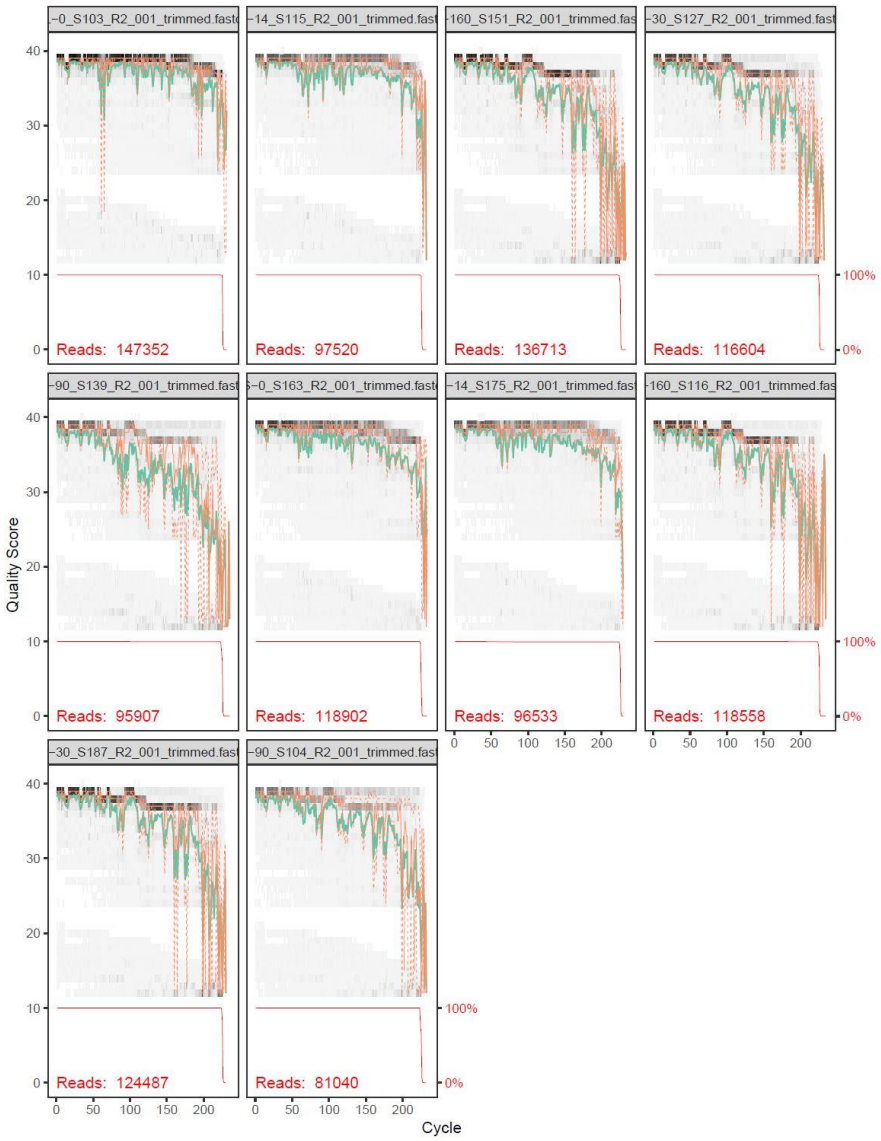

Figure 11. Quality plots of the reverse reads. Generated quality plots by the DADA2 plugin. The green line shows the median quality score, and the quartiles of the quality score distribution by the orange lines. The red line indicates the scaled proportion of reads that extend to at least that exact position.

7.6.3.2. **Filter and trim**

Unlike many other sequences, the ITS segments should not be trimmed, as they have a high length diversity that include mandatory biological information [39]. Therefore, it is not recommended to trim the sequences by length, otherwise information is permanently lost. Other filter parameters applied in DADA2 are as followed:

maxN=0, maxEE=c(2,2), truncQ=2, rm.phix=TRUE

maxEE is the maximum of expected errors per read and is a better filter than simply averaging quality scores of the sequences.

7.6.3.3. Learn the error rates

The DADA2 plugin for R makes use of a parametric error model to enhance the sequence quality. Each amplicon dataset contains a different set of error rates; the algorithm in DADA2 is able to recognize the unique error model from the data. By alternating between estimations of the error rates and inference of sample composition, they eventually converge to a consistent solution. Like many machine-learning algorithms, it starts with an initial guess, for which the maximum possible error rates in the data are used. Arguing that the most abundant sequences are correct, and all the rest are errors [56].

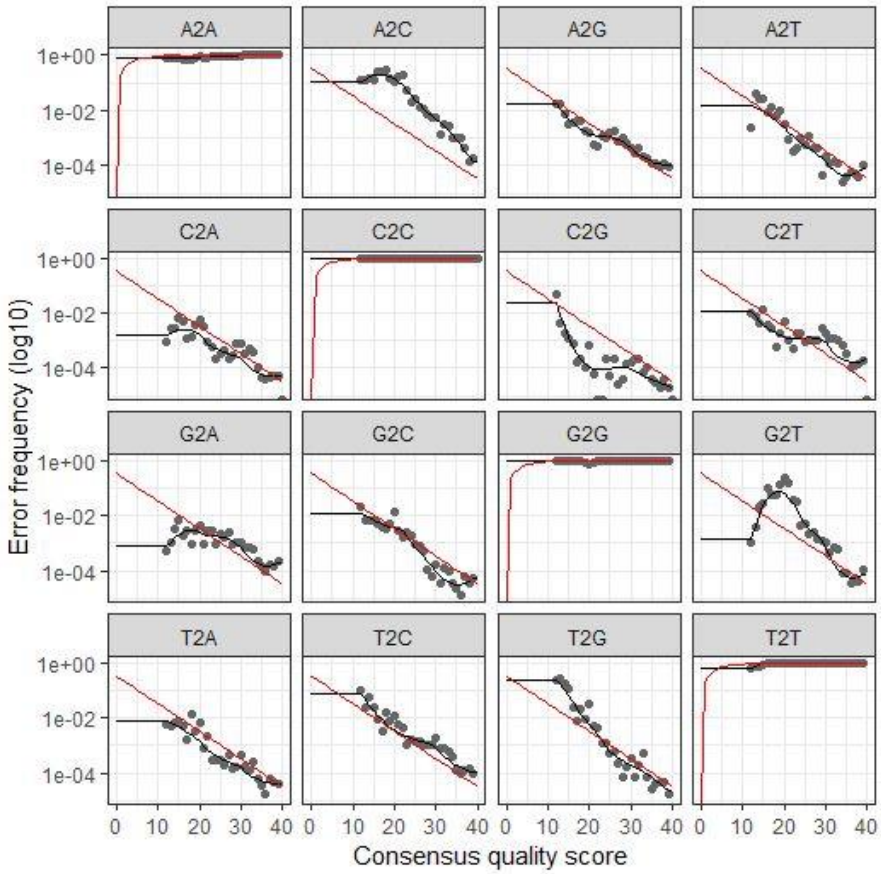

Figure 12. Error frequencies of the forward reads. A plot generated in DADA2 indicating the error frequency for each substitution that occurs in the forward reads. The red line shows the expected error rates under nominal definition of the Q-score while the black line represents the observed error rates [84].

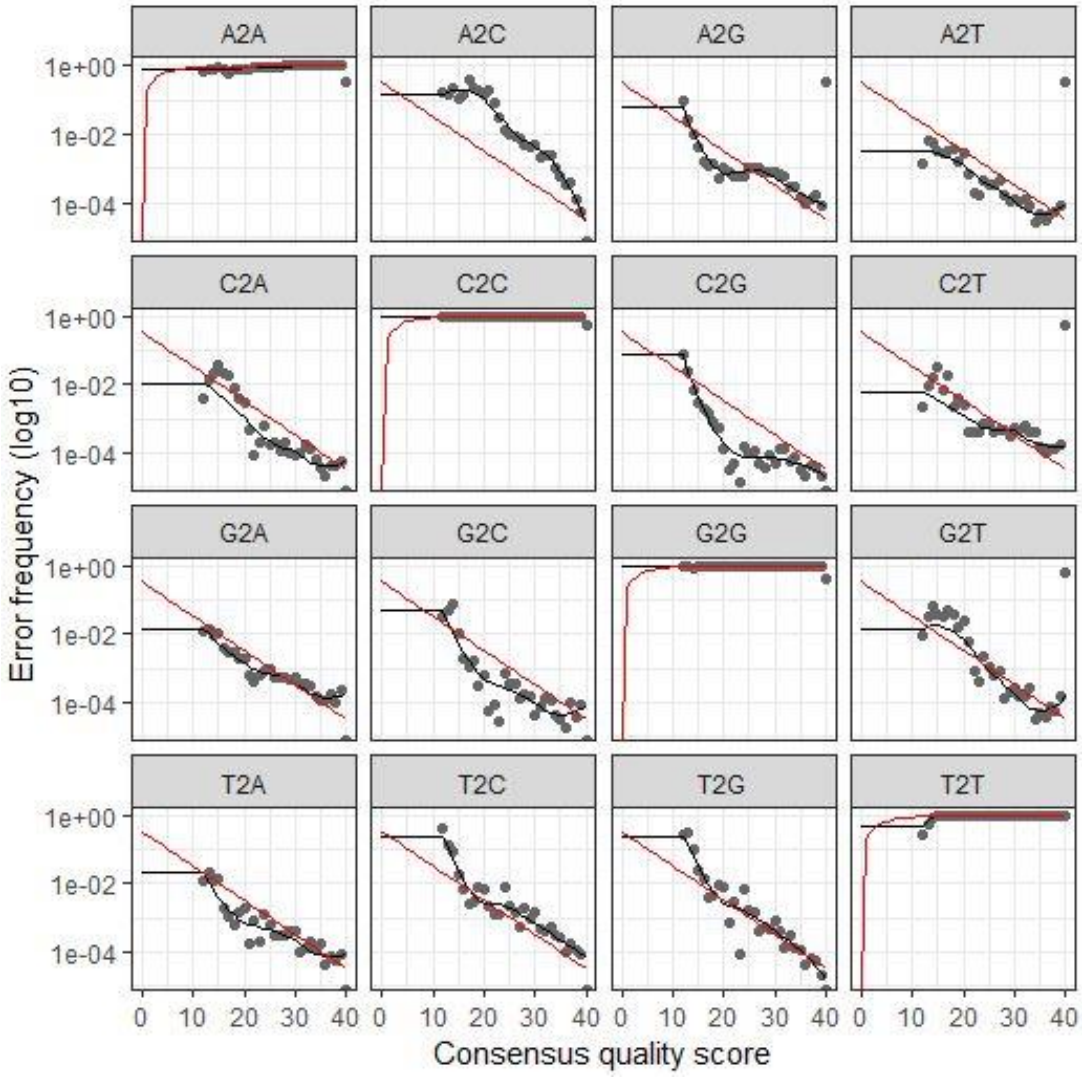

Figure 13. Error frequencies of the reverse reads. A plot generated in DADA2 indicating the error frequency for each substitution that occurs in the forward reads. The red line shows the expected error rates under nominal definition of the Q-score while the black line represents the observed error rates [84].

Figures 12 and 13 show possible base transitions like A→C or A→G. The dark dots represent the witnessed error rates for each consensus quality score. The estimated error rates after convergence of the machine learning algorithm is represented in the black line. Error rates expected under nominal definition of the Q-score is shown in red.

7.6.3.4. **Dereplicate identical reads to reduce computational time**

Reads that are entirely identical will be reduced to save computational power and reduce processing time for the further applications.

Plant	Sample	Total	Unique	Plant	Sample	Total	Unique
	A 0	113052	13443		B 0	142120	11188
A	A 14	91534	10947	B	B 14	113715	7906
	A 30	14708	14708		B 30	97787	17889
	A 90	72080	9602		B 90	67192	9330
	A 160	101980	14409		B 160	113715	14547

Table 2. Amount of unique sequences after dereplication step.

7.6.3.5. **Merging**

Combines the overlapping forward and reverse reads to obtain a complete sequence.

7.6.3.6. **Core sample inference algorithm**

The core sample inference algorithm constructs an amplicon sequence variant table (ASV) that records how often an exact amplicon sequence variant is observed in each sample. The outcome has a higher resolution than the common OTU table produced by traditional computing methods. Sequences that are the possible result of nonspecific priming indicated by too long or too short sequences are removed from the generated sequence table. Only sequences between 230-398 bp were used for further analysis.

7.6.3.7. **Remove chimeras**

A chimera is a single DNA sequence originating from multiple transcripts or sequences. They are generally considered a contaminant, as a chimeric sequence can be interpreted as a novel sequence while it is in fact an artefact. After application of the denoising algorithm, identification of chimeras is simpler. In this step, the software tries to reconstruct the exact sequence by combining the left and right segments from two or more abundant sequences. In this analysis 139 out of 406 sequences were identified as chimeras, in total it was less than 9% of the overall read counts.

7.6.3.8. **Final output**

After processing the sequences through the various steps to enhance the quality and ensure their uniqueness, DADA2 provides a compact summary of the number of sequences that remained after each step.

Input	Filtered	DenoisedF	DenoisedR	Merged	Nonchim	Final
A 0	118902	113052	112799	112942	103605	97923
A 14	96533	91534	91154	91419	80438	79773
A 30	124487	108339	108277	108269	41235	40170
A 90	81040	72080	72050	71968	42442	38635
A 160	118558	101980	101964	101916	37236	35536
B 0	147352	142120	141780	142070	132269	130174
B 14	97520	93692	93648	93637	89041	86270
B 30	116604	97789	97744	97705	44360	40472
B 90	95907	67192	67137	67081	33446	32134
B 160	136713	113715	113651	113630	41687	41000

Table 3. Summarized information about sequences obtained during the processing. Each row resembles the sequences gathered from one time point of ripening. Numbers represent the sequences that remain after each process mentioned in the top row of the table.

7.6.3.9. **Assigning taxonomy**

To assign the taxonomic traits to the pool of sequences the latest available UNITE reference database was used. The set of sequences comes in fasta format and the file itself is named “sh_general_release_dynamic_s_01.12.2017” and was made publicly available in 2017 via the Unite homepage (https://unite.ut.ee/repository.php).

7.6.3.10. **Tree**

After assigning the taxonomy to the sequences, an alignment was made to create a phylogenetic tree. By using FastTree, (http://www.microbesonline.org/fasttree/) a tree file was generated. The tree_file was loaded into R studio via the plugin “ape” version 5.3 that was capable to read, write and manipulated phylogenetic trees.

7.6.3.11. **Phyloseq 1.26.1**

For further analysis the “phyloseq” object was constructed. Phyloseq is a plugin available for R that can handle and analyse high-throughput microbiome data. It provides a set of tools to facilitate the analysis and graphical display of microbiome census data.

8. Results

8.1. Cultivation

A total of 62 VB cheese fungal isolates were obtained, and their taxonomic affiliation (18S rRNA gene, ITS2 region) was determined by comparing sequences to the closest related type strains in the NCBI and UNITE databases [44, 45]. The most of the isolates belonged to filamentous fungi, *Scopulariopsis* sp. (39%) and *Aspergillus* sp. (35%). Other isolates were identified as *Debaryomyces*, *Penicillium*, *Alternaria*, *Acremonium* and *Candida*.

Number of isolates (n=62)	Best Blast/UNITE hit Accession number	Sequence identity (%)
24	*Scopulariopsis sp.*, KX924023	99.70%
22	*Aspergillus* sp., MK605984	99.36%
8	*Debaryomyces sp.*, KY103234	99.41%
5	*Penicillium sp*, CBS115506	99.02%
1	*Alternaria sp*, KF573995	99.66%
1	*Acremonium* sp., KF669512.1	99.37%
1	*Candida sp.*, MK394125	100%

Table 4. VB cheese fungal isolates.

8.2. qPCR

Total fungi DNA was quantified from each of the extracted samples (n=200) using the 18S rRNA TaqMan qPCR assay [47].

Plant	Days	Mean	Median	Min	Max	IQR	Shapiro
A	0	2.19E+08	2.20E+08	4.20E+07	3.62E+08	6.35E+07	0.44
	14	6.67E+06	5.74E+06	1.66E+06	1.65E+07	6.52E+06	0.02
	30	5.66E+06	4.93E+06	2.31E+06	1.69E+07	2.26E+06	0.00
	90	2.34E+06	2.11E+06	1.42E+06	4.76E+06	9.83E+05	0.00
	160	1.99E+06	2.10E+06	8.68E+05	3.36E+06	1.00E+06	0.76
B	0	1.97E+07	7.52E+06	1.97E+06	8.77E+07	2.80E+07	0.00
	14	1.15E+06	5.81E+05	1.12E+05	8.94E+06	8.30E+05	0.00
	30	3.14E+06	7.94E+05	1.75E+05	1.62E+07	4.82E+06	0.00
	90	4.51E+07	5.11E+07	3.91E+06	9.83E+07	5.47E+07	0.10
	160	1.01E+07	6.83E+06	3.35E+06	3.87E+07	8.23E+06	0.00

Table 5. Fungal cell equivalents (FCEs) per 0.5 g rind cheese of two cheese production plants (A and B) at day 0, 14, 30, 90, and 160 of ripening. IQR: Interquartile range.

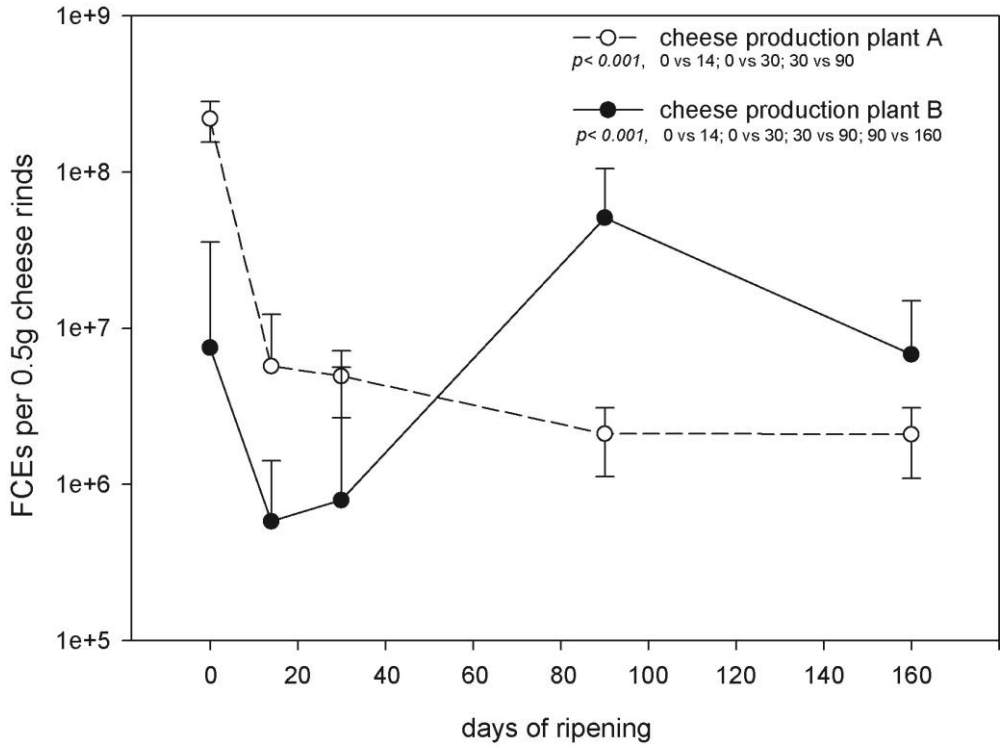

Figure 14. Abundance of cheese rind fungi during ripening of VB in two cheese production plants (A, B) determined by qPCR. Fungal cell equivalents (FCE) per 0.5g of cheese rinds are shown. The graph shows median and interquartile ranges for 20 samples for each analysed point of ripening (0, 14, 30, 90 and 160 days), for each plant (A, B).

The results of 18S rRNA gene TaqMan qPCR assay confirmed that fungi are abundant on the VB cheese rinds (n=200) throughout the ripening time (160 days) (Figure 14). Overall, a higher abundance was found in plant A as compared to B (Median 2.20e+08 and 7.52e+06, respectively). Similar to plant B, FCEs in plant A decreased significantly during the first 14 days ($p>0.001$) and remained relatively constant in plant A until the end of the ripening process. In contrast, FCEs in plant B increased significantly from 7.94e+05 at day 30 to 5.11e+07 at day 90 ($p>0.001$), and finally decreased significantly ($p>0.001$) at day 160.

8.2.1. Kendall rank correlation coefficient

No relationship between the two cheese production plants (A, B) were found (Kendall's Rank correlation Tau-B), thus obtained qPCR data were analyzed separately.

8.2.2. Wilcoxon signed rank test

Statistical differences between ripening points (0, 14, 30, 90, 160) and cheese production plants (A, B) are shown in Table 6.

Plant	0 vs. 14	0 vs. 30	14 vs. 30	30 vs. 90	90 vs. 160
A	**<0.01**	**<0.01**	0.22	**<0.01**	**<0.01**
B	**<0.01**	**<0.01**	0.67	**<0.01**	0.19

Table 6. Fungal FCEs comparison of different ripening times. Statistical differences between subsets within the same cheese production plant (A, B) and during ripening points (0, 14, 30, 90 and 160) were calculated using the Wilcoxon Signed Rank Test. P-values lower than 0.05 are interpreted as statistically significant and are highlighted in bold.

8.3. Illumina-preliminary results

After quality control of the raw sequencing reads 1,133,616 ITS reads (48.6%) remained, clustering into 92 amplicon sequence variants (ASVs).

8.3.1. Rarefaction curve

Rarefaction is a technique to estimate the species richness in the samples. Species richness is the number of different species in a defined sample or ASV´s. When the amount of species identified is nearly enough to describe the sample the curve will converge, if the species richness is still increasing as more samples are added the data is not sufficient to estimate the richness for the full population. The rarefaction curve in Figure 15 is generated in “ggplot style” and the colour scheme was set to default.

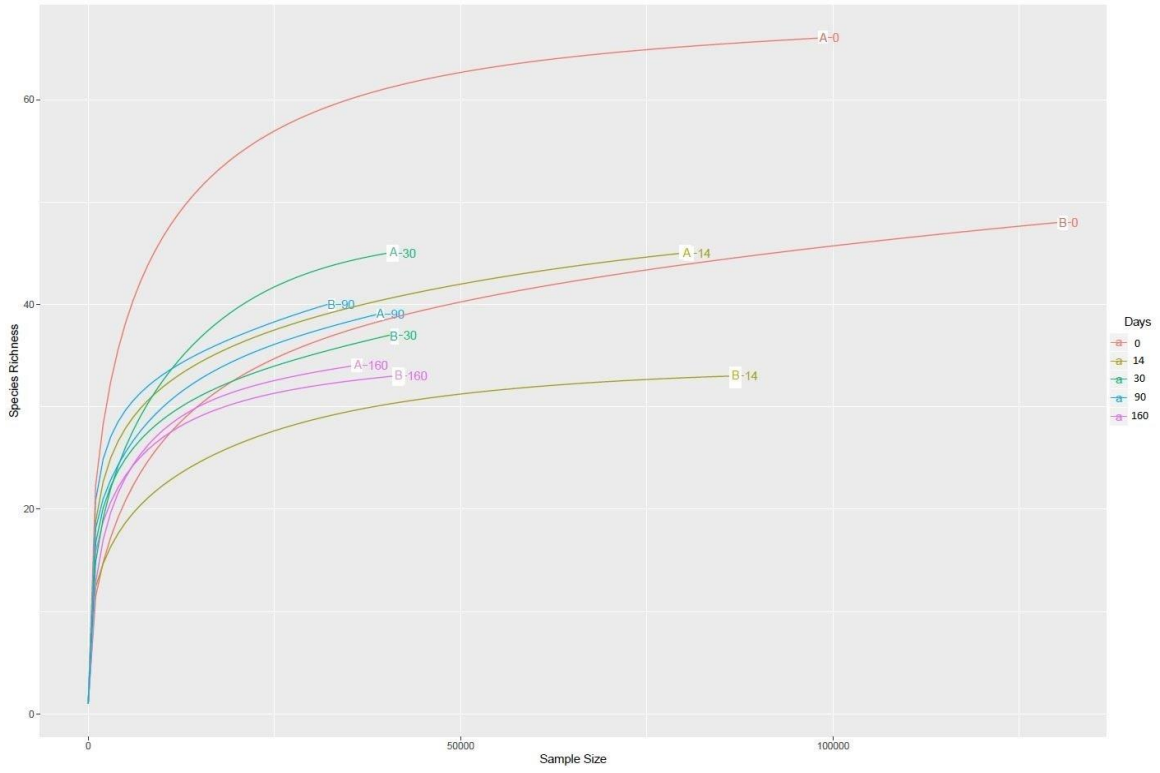

Figure 15. Rarefaction curves of the cheese rind samples. The y-axis shows the richness of species that would be expected to be found in the sampling size shown on the x-axis.

Determination of the microbial community structure helps to understand relationships and niche portioning. Alpha diversity analyses done by Chao1 and inverse Simpson values represent comparative information combining the two main components of community diversity the richness and evenness. The Shannon index measures how evenly the organisms are distributed in each sample [57].

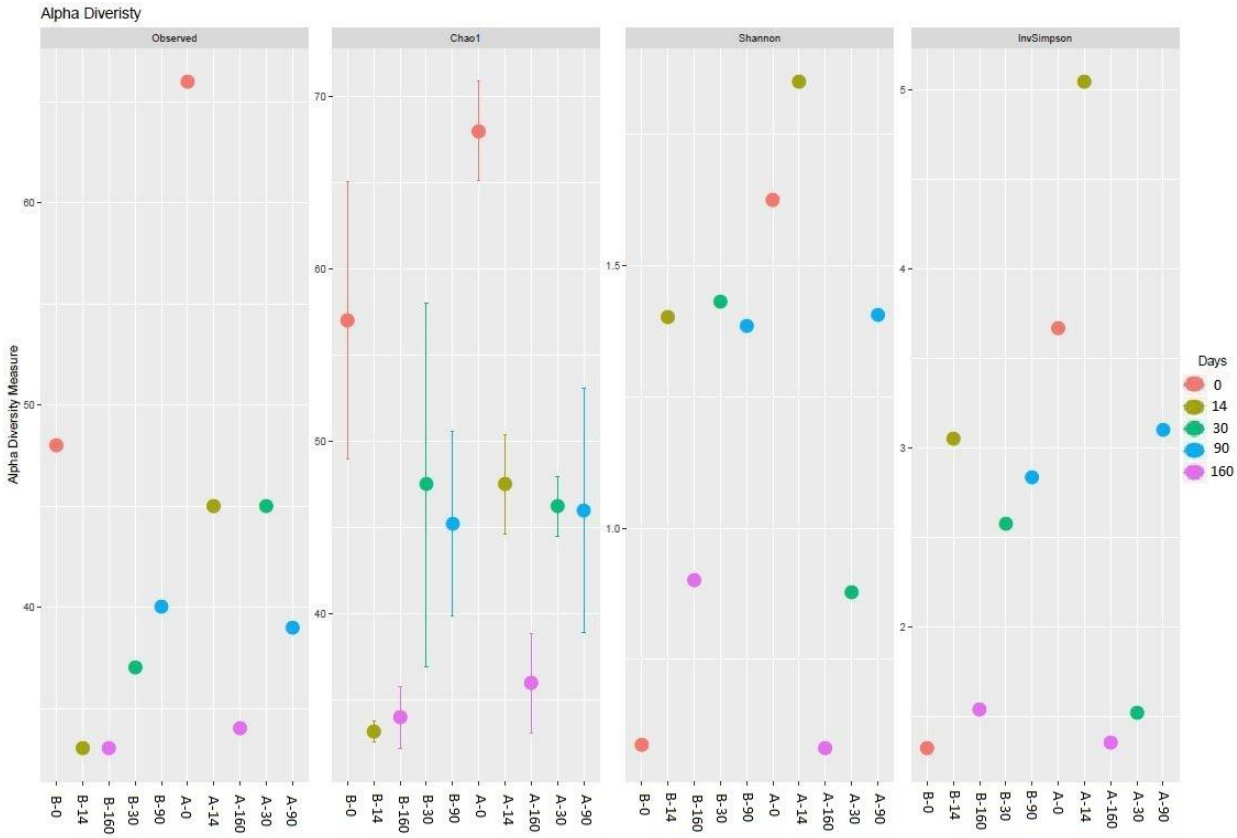

Figure 16. Alpha diversity indices (richness, Chao1, Shannon and inverse Simpson). Larger values on the y-axis indicate a higher alpha diversity. X-axis, samples.

The highest number of different species was observed on day 0 in plant A. It is the highest diversity measured followed by plant B of the same day. The lowest diversity was measured on day 14 and 160 of plant B. Chao1 shows the relation between species that occur just one time (singletons) and the once found twice (doubletons). Both plants show the highest abundance of singletons and doubletons on the day 0. When the Shannon-index is applied cheese plant A shows the highest diversity and abundance in week 2 of the ripening process, followed by the production day of the cheese wheel itself. B14, B30, B90 and A 90 show an intermediate level of abundance and diversity while B0 and S160 represent the lower outcome of this index. When the inverse Simpson diversity estimator is applied the most significant difference is a decrease of day 30 and 90 in plant B. Using the former index both values are close to the level of day 14, and with this estimator their diversity is slightly below it.

8.3.2. PCoA plot

PCoA stands for Principal Coordinate Analysis and plots the fungal beta-diversity based on the Bray-Curtis dissimilarity. This method named after its inventors is used to quantify the compositional dissimilarity between two different sites or plants in this case. The index

of dissimilarity is based on counts at each site and bounded between 0 and 1, whereas 0 means the two sites have the exact same composition and share all species and 1 stands for sharing not a single species.

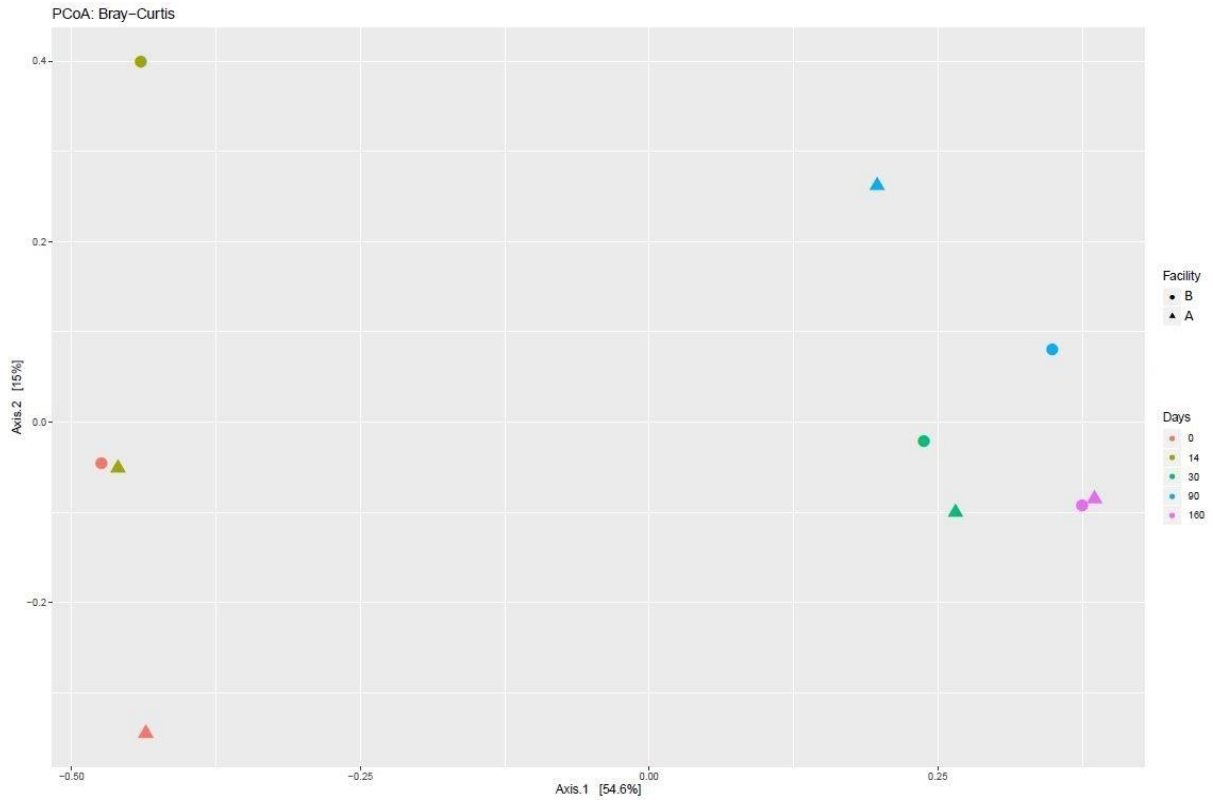

Figure 17. Bray-Curtis dissimilarity plot. Percentages shown at the axis description are percentages of variation explained by the components. The dots represent values of plant B and the triangle plant A. The different sampling time points are indicated by the colour of the symbols according to the figure's legend.

Fungal communities evolved through time similarly in VB rinds from both plants, and so the samples clustered together regarding the ripening time point, with the samples corresponding to the latest ripening times (30, 90,160 days) clearly separated to the early ones (0, 14 days).

8.3.3. Relative abundance of fungal families in VB cheese rind samples

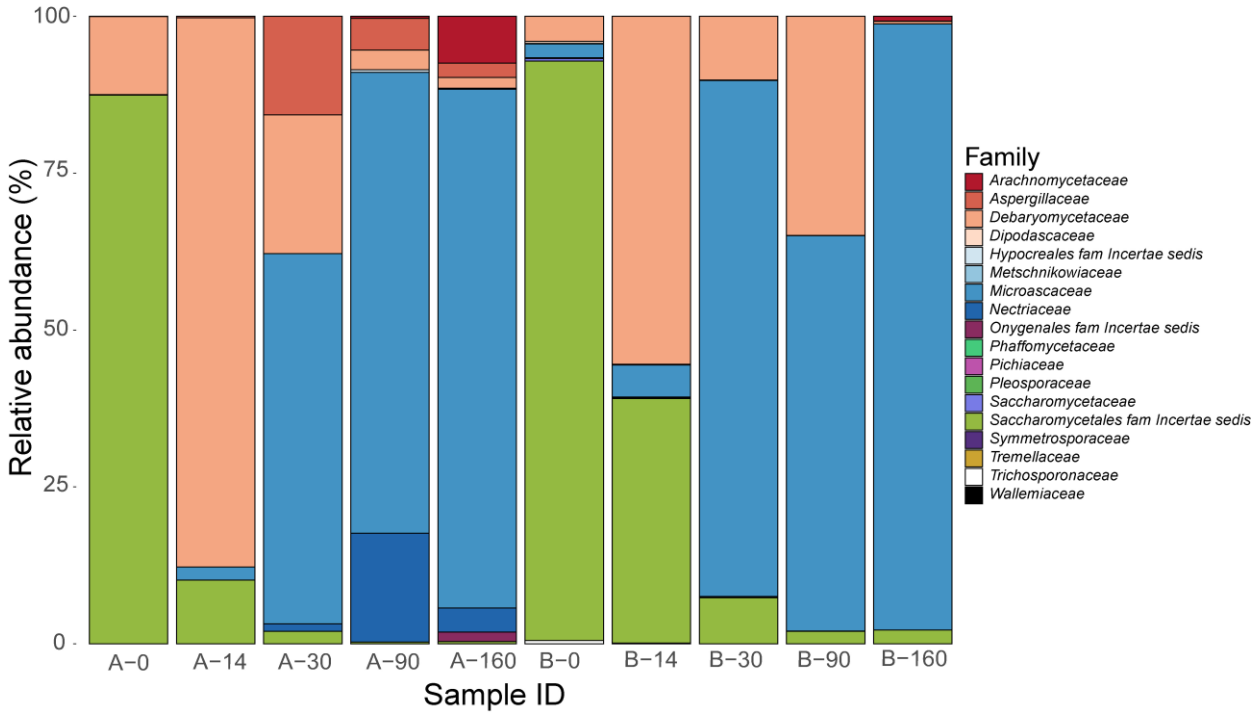

Figure 18. Relative abundance of fungal families in VB-rinds during ripening. Abbreviations: plants (A, B); days of ripening (0, 14, 30, 90, 160).

The Illumina sequencing data analysis gave the first insight into the VB-fungal profile in cheese rinds. A total of 18 families were identified throughout both plants (A, B). *Saccharomycetales fam Incertae sedis* was dominant during the first 14 days and decreased throughout the rest of the ripening time. While *Microascaceae* was lower at day 0 and 14, it increased thereafter in both plants. *Debaryomycetaceae* increased at the first day of production and then subsequently decreased during the ongoing ripening. The most abundant genera found were *Scopulariopsis*, *Candida*, *Debaryomyces*, *Yamadazyma*, *Fusarium* and *Penicillium*, which represented 97.8% of the entire fungi identified. The relative abundance analysis revealed a large shift in the fungal community between day 14 and 30.

9. Discussion

In this master thesis, fungal communities from a total of 200 Vorarlberger Bergkäse (VB) wheels, including two plants (A, B) and 5 ripening time points (0, 14, 30, 90 and 160 day of ripening), were investigated, using culture-dependent (cultivation) and culture-independent methods (qPCR and high-throughput sequencing).

9.1. Cultivation approach

It is known that the major limitation of most microbial communities is the difficulty to isolate all abundant taxa in cultivation approaches [58]. They are strongly biased through the selection of cultivation media and the cultivability of microorganisms. Many studies reported, that a large uncultivable fraction of species remained uncultivable [59]. In this study, it was possible to isolate 62 fungi from 7 different genera. We isolated *D. hansenii,* as one of the most abundant yeast on the VB cheese rind in this study. The occurrence of *D. hansenii* in raw milk and cheese has been confirmed by many studies [60-62] and has been found on Fontina and various Austrian cheeses [15, 59, 63, 64]. *Debaryomyces* has been recognized as the most common organism isolated from the cheese surfaces [65]. Another isolated yeast, *Candida,* dominated rind communities at the early time points and several species of *Candida* have been frequently found on rind of European smear-ripened cheeses [66-68].

Yeasts, such as *Candida* and *Debaromyces* play a major role in the fermentation process of dairy. Due to their ability to grow at low temperatures and in high salt concentrations, they can sustain the ripening environment. Some physiological and biochemical characteristics including the trait to utilize lactose or galactose and high proteolytic and lipolytic activity enhances their performance during ripening. Texture development and production of various aroma compounds during ripening through secrete enzymes are also major contributions [69, 70].
The presence of *Scopularopsis*, *Aspergillus*, *Alternaria* and *Acremonium* on the surfaces of cheeses has been reported previously [61, 71, 72]. The results are consistent with the recent studies based on surface ripened Danish cheeses, where *Scopularopsis* has been found [62]. The presence of filamentous fungi has been detected in a high number of cheeses and in raw milk [73]. Due to their high proteolytic activity resulting in ammonia production, they are often subjected to spoilage. Arsenical compounds like diethylarsine derive from them and result in a very characteristic garlic-like odor [62].

In the VB cheese production, no fungi as starter cultures are used. Therefore, the cheese producing environment may act as a source of natural microbial inoculation, especially in traditionally manufactured products like VB-cheese. Studies have been shown that the majority of the cheese rind community would originate from environmental sources [74, 75].

As mentioned above, conventional culture-dependent approaches are limited and are not able to depict the fungal diversity of the cheese habitat. Therefore, the usage of molecular techniques is mandatory to obtain a high resolution of the species within the samples.

9.2. Non-cultivational approach

Preliminary analysis of the Illumina data presented here provides the first insights into the mycobiome of VB-cheese rinds. The results should be interpreted as preliminary, since different pipelines for fungal data will also be applied to get the best quality of the ITS2 amplicon sequences.

In a previous study, the fungal composition of the rind microbiota of VB-rind cheese was assessed using cloning and further Sanger-sequencing [16]. Therefore, the present study can only be compared to a very limited extent [16, 75]. The use of internal transcribed spacers (ITS) region of the rRNA, being a primary fungal DNA barcode and thus is used for taxonomic identification [39].

The analysis revealed distinguishes composition of fungal communities in two cheese producing plants. A high relative abundance of *Debaryomyces* (*Saccharomycetaceae*) and *Scopularopsis* (*Microascaceae*) in this study was consistent with a previous research of Schornsteiner et al. [16]. As mentioned above, *Debaryomyces* is being highly abundant yeast in smear-ripened cheeses, and the cheese processing environment and brine have been recognized of their important source [28, 74, 76-78].

The occurrence of filamentous fungi (molds) can originate from raw milk or maybe introduced during cheesemaking either from the environment or ripening cultures [79]. Some filamentous fungi which occur in raw milk, are not very likely to persist and grow in cheese due to the biochemical composition and the hurdle effect exerted by the cheese microbiota [80]. The encountered filamentous fungi therefore may originate from the ambient air, the dairy environment or the ripening plant.
During the early stage of ripening, filamentous fungi produce a large number of hyphae to increase access to the cheese matrix nutrients [79].This is in accordance with the recent study, where *Scopularopsis* was absent or in very low abundance at the beginning of ripening. Similarly to other recent studies [81-83], we enabled the detection of further highly salt-tolerant species, such as *Yamadazyma (Debaryomycetaceae).*

Outside of the differences between both plants, some patterns were similar and common for the fungal progression during ripening process. The decline of *Saccharomycetales fam Incertae sedis* within the first 30 days is mostly due to a depletion of lactate [84]. Correspondingly, in this work *Debaryomyces* was the most abundant at 14 and 30 days of ripening. An increase of the pH during the early stages of ripening is caused by the

production of alkaline metabolites from yeasts. This changes lead to a shift in the microbial community towards less acid- and salt- tolerant bacteria on the surface [85].

9.3. qPCR assay

To determine the abundance of fungi during the ripening on the VB cheese rinds, a qPCR assay was performed. The FungiQuant assay showed a high specificity of the used primers and has been supported and recommended for fungal quantification [48, 49]. To our knowledge, there is no universal method to quantify total fungi on cheese rinds. Only few quantitative data investigating the cheese fungi during ripening are available, focusing on the abundance of certain phylotypes e.g. *Geotrichum*, *Penicillum* and *Debaryomyces* [66, 86-88]. Moreover, fungal ribosomal RNA operon copy numbers vary and therefore is a strong bias towards with more copies [89].

Quantitative data on the FCE showed relatively high numbers at the first day of cheese production, which suggested that fungi may originate from raw milk or milk processing surfaces used for cheese production.

Further comparative studies of bacterial and fungal community and single community members are required to evaluate and validate the value of qPCR and dPCR technologies in the cheese rind applications. The usage of additional or alternative markers like the *β*-tubulin (*tub2*), the translation elongation factor 1-α (*tef1α*) or the second largest subunit of ribosomal polymerase II (*rpb2*) could improve the fungi quantification in further studies [90-92].

10. Conclusion

Fungi are abundant on VB rinds throughout ripening time and differed between two cheese production plants. The microbiota of VB rinds evolved throughout ripening and different fungi were identified in both plants at specific ripening periods, regardless the ripening plant investigated. In general, ripening cheese communities formed distinguished groups between younger (until 30 days of ripening) and older cheese rinds. Molecular-based technologies (HTS, qPCR) are valuable for highlighting the existence of previously unexpected cheese rind microbial communities. The knowledge of the cheese rind composition allows us to better understand the VB cheese-ripening process and potentially enable the targeted approaches to be improved, enhancing the quality of the product.

11. Outlook

Further studies are mandatory to characterize cheese-associated microbiota, present in the VB-rinds during ripening process. The interactions between fungi and bacteria on the cheese surface are complex and not yet fully understood. Further studies on cheese core and cheese rinds are needed to investigate the relationship between these communities and changes in the chemical characteristic. Metatranscriptome (RNA-based) analysis would highlight the expression of functional genes within the identified communities to reveal their potential functions of the rind microbiota.

12. List of figures

13. List of tables

14. References

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