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A study of pro-technological and spoilage yeasts in the food industry

Ph.D. Thesis

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Chapter 1- Introduction

Background and aim

Yeasts are unicellular eukaryotic microorganisms classified as members of the fungus kingdom. They are one example of the earliest exploited organisms. In fact, archaeologists have discovered evidences showing that humans had exploited yeasts to leaven bread and ferment beverages since 7.000 BC (Sicard and Legras, 2011). This ancient, close relationship between yeast and food is linked to the process of fermentation. This is one of the oldest forms of food processing and preservation. Yeasts are microorganisms predominant in several fermented foods and beverages, both of animal or vegetable origins, such as milk derivatives, leavened products and alcoholic beverages. They can be selected and used as starter cultures to produce industrial food or they can be responsible for spontaneous fermentation both alone and together with lactic acid bacteria, acetic acid bacteria, mycelial fungi. Yeasts have a significant impact on food quality improving taste, flavour, texture, nutritive values, reducing anti-nutritional factors and promoting health properties. However, yeasts are also known as agents that can cause spoilage of foods and beverages, resulting in reduced appealing for the consumers for products characterized by objectionable odour, appearance, taste or texture. This double role of yeast in food industry is often related to strain-dependent properties.

Pro-technological role of yeasts in food industry

Yeasts are well known for their ability to produce a wide range of fermented foods and beverages from different substrates. Moreover, yeasts can lead the food fermentation process to produce and use organic acids, to enhance nutritional properties of products and to reduce anti-nutritional factors and mycotoxins eventually present in the raw materials (Chaves-Lopez et al., 2014; Dung et al., 2006; Liu and Tsao, 2009). Yeasts can have also several applications in the functional food industry, bringing to different types of products and contributing to the production of nutraceuticals. Functional foods are defined as conventional foods that have demonstrated health benefits beyond their nutritional properties; nutraceuticals are purified food components with proven health benefits (Padilla et al., 2015; Rai et al., 2017; Rai and Jeyaram, 2017). Yeast living cells can be used as probiotics, while their cell wall constituents can have a nutraceutical value. Lastly, yeast can be able to release bioactive metabolites and/or enzymes responsible of food metabolites biotransformation (Padilla et al., 2015; Rai et al., 2016). Several authors, as a matter of fact, have reported yeasts producing specific enzymes, such as *Kluyveromyces lactis, Kluyveromyces marxianus, Saccharomyces cerevisiae*, which help in production of substrate-derived bioactive compounds such as peptides, oligosaccharides and free polyphenols (Chua et al., 2017; Padilla et al., 2015; Rai et al., 2016). The most important goal in the development and consumption of functional foods is gut health. For this purpose, three different types of food ingredients can be used: living microorganisms (probiotics), non-digestible carbohydrates (dietary fiber and prebiotics), and bioactive molecules that are plant secondary metabolites such as phenolic compounds (Puupponen- Pimiä et al., 2002; Puupponen- Pimiä et al., 2005).

Probiotics

World Health Organization defines probiotics as "living microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO and WHO, 2002). Generally, a lower limit of 10^9 CFU per dose is used to obtain the benefit, even if it depends by the strain, the food and the health effect (Forssten et al., 2011). To be considered a probiotic, a microorganism must satisfy several criteria. It must be innocuous, it must not cause illness or be toxic to the host, it must be in high amounts in the product with which it is administered and maintain its viability during its shelf-life, and it must survive to the gastrointestinal transit (Hill et al., 2014; Tripathi and Giri, 2014; de Vrese and Schrezenmeir, 2008). The survival of the microorganism is linked to its different capabilities such as growth at low pH, resistance to digestive enzymes and bile tolerance (Fadda et al., 2017). Yeasts used as probiotics in food industry holds several advantages in comparison to bacteria. In fact, they are bigger than bacterial cells, they are resistant to antibiotics, and have not been reported for transfer of genes related to antibiotic resistance (Rai et al., 2018). Yeast species that have shown probiotic properties are *S. cerevisiae*, *Saccharomyces cerevisiae var. boulardii*, *K. marxianus*, *Kluyveromyces lactis*, *Debaryomyces hansenii*, *Torulaspora delbrueckii*, *Candida krusei*, *Yarrowia lipolytica*, *Pichia fermentans* and *Pichia kudriavzevii* (Rai et al., 2018). Among these species, *S. cerevisiae var. boulardii* is the most studied and it is the only one used for therapeutic and preventive purposes (Rai et al., 2018). Several studies have indeed shown that *S. cerevisiae var. boulardii* confers beneficial effects against various enteric pathogens using different mechanisms. Its effectiveness as probiotic has been demonstrated in the prevention of different types of diarrhea such as Antibiotic-associated diarrhea (ADD) and traveler's diarrhea (Surawicz et al., 2000; Kotowska et al., 2005; McFarland, 2007). Notably, the administration of *S. cerevisiae var. boulardii* can prevent AAD in both adults and children. Moreover, this species has been shown to be useful in the treatment of patients with irritable bowel syndrome (IBS) and Crohn's disease (Maupas et al., 1983; Plein and Hotz, 1993).

Functional food ingredients: yeast cells wall components

β-glucans are known to improve the immune system, to low cholesterol levels in blood and they have exhibited anti-inflammatory activity (Vieira et al., 2016). Considering that cell wall of *S. cerevisiae* is an important source of β-glucans, in 2011 the European Food Safety Authority (EFSA) has approved the application of *Saccharomyces* β-glucans (yeast beta-glucans) as a novel food ingredient, proposing a concentration limit ranging from 50 to 200 mg (EFSA, 2011). These β-glucans has demonstrated to modulate immune response and to enhance the INF-γ, suggesting its ability to stimulate the immune system (Javmen at al., 2015). Moreover, mannan extract from cell wall of *S. cerevisiae* can be added to yogurt to increase the vitality of probiotic bacteria (Al-manhel and Niamah, 2017) while mannan from *K. marxianus* has demonstrated to have antioxidant activity (Galinari et al., 2018)

Potential nutraceutical role of bioactive metabolites from yeasts

Yeasts can be able to produce several bioactive components, which form an integral part of the functional food industry such as γ-aminobutyric acid (GABA), carotenoids and folate (Chen et al.,2016; Greppi et al., 2017; Han and Lee, 2017). In fact, fermented foods and beverages by yeast have shown to possess several bioactive metabolites, improving the functionality of the final product. For example, a study has shown that a *S. cerevisiae* strain isolated from traditional Korean Bokbunja wine is a potential candidate to produce functional wines enriched with GABA (Song and Baik, 2014). Carotenoids are natural pigmented bioactive compounds, having a potential application in the functional food industry as they are known for preventing oxidative stress – related diseases (Chen et al., 2016; Mannazzu et al, 2015). Folate (vitamin B_9) is one of the essential cofactors in several biochemical reactions and its deficiency is becoming a world-extended problem (Korhola et al., 2014; Greppi et al., 2017). In fact, mammals do not have the ability to synthesize folate and they are

consequently dependent on its absorption through the diet (Scott et al., 2000). Folates have a role in the prevention of neural tube defects in the fetus (Bailey et al., 2003; Cordero and Berry, 2008), and a sufficient folate intake can reduce the risk of cardiovascular disease, cancer and Alzheimer's disease (Bailey et al., 2003; Ward, 2001; Duthie, 1999; Wang, 2002). The folate level can be augmented in fermented foods using an appropriate yeast strain and optimizing the growth phase and cultivation conditions for the selected strain. Folate content in white wheat bread leavened using a specific *S. cerevisiae* strain was found to enhance by 3–5 fold depending on culture conditions, in comparison to bread leavened with commercial baker's yeast (Hjortmo et al, 2008). In beer, the amount of folate enhances due to synthesis by yeast during the initial period of the fermentation. However, since yeast folate is intracellular, it will be eliminated from the beer except for those with a secondary fermentation phase (in the bottle) that, keeping the yeast cells, contain a higher level of folate (Jägerstad et al., 2005). This is the typical manufacture process of a craft beer. Kefir, a fermented milk beverage originated in Eastern Europe and regarded as a natural probiotic product, contains high folate content (Zubillaga et al., 2001; Patring et., 2006). Kefir grains, that are responsible of the milk fermentation, have a varying and complex microbial composition including species of lactic acid bacteria, acetic acid bacteria and yeasts. These yeast strains showed a high folate producing capacity (Moslehi-Jenabian et al., 2010). Therefore, a selection of appropriate yeast strains as starter cultures in fermented foods, appears to have a positive impact on consumers particularly in developing countries where the vitamin intake is generally low.

Yeasts role on bioavailability of nutrients

Yeasts can be also applied for the pre-treatment of foods to reduce the phytate contents or utilized as food supplement to hydrolyze the phytate after digestion. Phytate is the primary storage form of phosphorus in mature seeds of plants and it is particularly abundant in many cereal grains, oilseeds, legumes, flours and brans (Moslehi-Jenabian et al., 2010). Phytate has a strong chelating capacity and forms insoluble complexes with divalent minerals of nutritional relevance such as iron, zinc, calcium and magnesium (Lopez et al., 2002; Maga 1982; Vohra and Satyanarayana 2003). Humans lack the required enzymes for degradation and dephosphorylation of the phytate complex; therefore, in food processing, its degradation can be catalyzed either by endogenous enzymes, naturally present in cereals, or by microbial enzymes produced by yeasts or/and lactic acid bacteria naturally present in flour or added as starter cultures (Türk et al., 1996). Improved adsorption of iron, zinc, magnesium and phosphorus can be achieved thanks to the degradation of phytate during food processing or by degradation of phytate in the intestine (Sandberg, 1991; Barbro et al., 1985; Sandberg et al., 1982). The study of phytase activity of yeasts in bread making has demonstrated that a *S. cerevisiae* strain with a high-phytase content, may be suitable to produce food grade phytase to be used in food production (Veide and Andlid, 2006).

Yeast spoilage activity in food industry

Yeast spoilage of foods or beverages can be defined as "growth of yeast in a food, sufficient to cause an alteration in that food, perceptible to a consumer, and liable to cause dissatisfaction, complaint, or rejection of that food by the customer" (Querol and Fleet, 2006). So, a yeast spoilage isolated from a spoiled product, and re-inoculated back into sterile food of the same variety will grow and cause identical symptoms of spoilage. The consequences of yeast spoilage can be different, but all of them

are the result of yeast growing in the food and consequent metabolic activity. The symptoms of spoilage are many and varied such as objectionable odor, flavor, appearance (turbidity, swelling, slime formation, discoloration), taste or texture, or a combination of these; not all of them may be obvious to all consumers and the food may be eaten (Fleet, 2011). However, generally consumption of yeast cells is not injurious to human health but part of a normal, healthy human diet. Indeed, unlike bacteria and viruses, yeasts are rarely associated with outbreaks of foodborne gastroenteritis, intoxications or other infections. Yeasts are not infectious organisms, but some species such as *Candida albicans* and *Cryptococcus neoformans* are opportunistic pathogens that cause a range of cutaneous, respiratory, central nervous system and organ infections, as well as general fungemia in individuals with weakened health and immune systems (Hazen and Howell, 2003). The raising frequency of these individuals in the community has led to an increase in reporting of yeast infections. Moreover, an increasing number of yeast species has been implicated, including many found in foods (e.g. *S. cerevisiae, Debaryomyces hansenii, Pichia anomala, Rhodotorula* spp. (Fleet and Balia 2006). Infections caused by *S. cerevisiae* are significant because of its extensive use in the food industry (Enache-Angoulvant and Hennequin, 2005; De llanos et al., 2006).

Microbial spoilage can occur at any stage of the production chain, from the raw material to the final packaged product. The consequences of a spoilage outbreak are considerable and include an important economic loss for the industry due to wasted product, recalling and disposal of the spoiled product. Moreover, a spoilage outbreak has a negative impact on the reputation of the company and on the image of the brand on consumers. Although bacteria and molds are the main responsible of spoilage, yeasts can make an important contribution. Yeast spoilage is perceptible to the customer, but considering that it does not concern public safety, food production companies are not required to advertise any spoilage incidents, and therefore relatively few outbreaks have been reported, in order to protect the company brands involved. Furthermore, yeast spoilage is often predictable: it occurs in products in which growing bacteria are retarded or prevented by the intrinsically properties of the

food and by the processing and storage of the product. Indeed, spoilage by yeasts is restricted to those foods with low pH, low water activity a_w (caused either by high salt or high sugar), low temperatures or containing weak acid as preservatives (Fleet, 2011). Over 120 different species of yeast are listed as being associated with food spoilage. However, most species show nonexistent or poor growth in properly processed foods (Barnett et al, 1983, Pitt and Hocking, 1985). It is important to note that the spoilage response may vary among strains within a species. Some strains could be spoilage microorganisms, while other strains may not be detrimental (Fleet, 2011).

Sources of contamination

To mitigate the effects of the natural presence of spoilage microbes in the food chain, it is important the application of standard procedures and good manufacturing practices. These precautions provide a reduction in the contamination, bringing it to acceptable levels. Moreover, the knowledge and understanding about the source of contamination can contribute to greater control over the development of undesirable species. Manufacturing facilities and equipment are the main sources of contamination for processed foods and beverages, due to the selective pressure exercised by the plant production environment to the yeasts. Other sources, such as raw ingredients, air, water and vectors, are also relevant.

Various types of vectors are responsible for dissemination of microbes in foods and beverages, primarily in vegetable foods. For examples, wasps have a crucial role in the preservation and dissemination of *S. cerevisiae* in wines (Stefanini et al., 2012), while *Drosophila* spp. contributes to the dissemination of *Brettanomyces* spp. (Dweck et al. 2015). Airflow can transfer and disseminate spoilage yeasts in the industrial facilities. Indeed, air can be a carrier medium and allows the microorganisms to travel either by adhering to dust particle or droplets or as a single particle (Curiel

et al., 2000). Also water used in the processing plants can be involved in the contamination of foods and beverages by spoilage yeasts.

The microbiological quality of raw materials is essential in the prevention and control of spoilage: higher initial contamination levels increase the risk of failure of technological control processes. As a rule, natural raw materials (e.g., fruits and sugar cane) have an adventitious microbial flora which is not active in foods after processing, being easily controlled through good manufacturing practices. However, when raw materials have already been processed (e.g., fruit concentrates, fruit juices, and sugar syrups), as in many foods of complex formulation (e.g., dessert yogurts, reconstituted fruit juices, and filled chocolates), contaminant yeasts are much more dangerous. This situation results from the enrichment of the contaminating flora with yeasts highly resistant to the environmental stress present during the processing of natural raw materials. Since detectable spoilage requires numbers of yeasts approximately 1×10^5–1×10^6 cells/ml (Ingram 1949), it follows that yeast spoilage requires growth of the yeast population. On grapes used for winemaking, diversity and abundance of the yeast population are very complex because they are related to multiple factors. Nevertheless, the main spoilage yeast in wine, *Dekkera/Brettanomyces bruxellensis,* (it can produce a concentration of volatile phenols over the sensory threshold), is absent or present in minimum amount on grapes, suggesting that the contamination occurs either during the winemaking process or in the storage areas and in the bottling line of wineries (Loureiro and Malfeito- Ferreira, 2003). In particular, the oak casks used in the maturation of wine are an important source of *Brettanomyces* contamination (Guzzon et al., 2017). In dairy products, yeasts are usually present in raw milk but are normally eliminated by pasteurization, so their presence, is caused by contamination during manufacturing. For example, small sediments of food such as milk or fruit on stainless steel surface increase the resistance of surface adherent spoilage yeasts against desiccation, disinfectants, and UV-C irradiation, contributing to the contamination of the final product (Shikano et al., 2017).

Factors comprising preservation systems

The microbial threat to any food is countered by the "preservation system". A preservation system can be regarded as a set of factors: characteristics intrinsic to the food, physical and chemical preservation, hygiene and packaging measures. Food itself can have features that help preserving it, such as low pH, low water activity, lack of nutrients or presence of essential oils or ethanol, high amounts of sugar or salt. Physical preservative measures include heat and pasteurization, chilled storage, carbonation or low oxygen concentration. Chemical preservation includes chemical preservatives such as sorbic, acetic and benzoic acids or sulphur dioxide. However, very few foods are protected by a single antimicrobial factor (Querol and Fleet, 2006).

Aim of the thesis

Summarizing, literature research shows that yeasts have a significant impact on foods. In fact, yeasts can improve the organoleptic properties of foods and promote health benefits. However, the number of studies regarding this research field is limited in comparison to other ones. On the other hand, yeasts are an important cause of spoilage in food industry, even if the attention has been focused on them only in recent years. Therefore, the research is limited in this field too, and it is necessary to enhance the knowledge about yeast spoilage through study and development of techniques aiming to detect and quantify these microorganisms in a quick and easy way. Study of natural alternative preservatives against yeast spoilage it is also important, as essential oils that have already demonstrated to have antimicrobial activity. The overall purpose of this Ph.D. research activity can be divided in two parts: i) study of yeasts with a pro-technological role in food industry ii) study of detection, control and prevention against yeast with spoilage activity.

Outline of the thesis

A brief description of the outline of this thesis follows.

In chapter 2, a study about nicotinamide riboside production in craft beers is discussed. It is pointed out the method used to detect and quantify the vitamin B_3 in craft beers and the active role of hop in nicotinamide riboside production. This study could be the first step to produce a low alcohol beer with a high vitamin B_3 content.

The microbial characterization of kefir grains from Bosnia and Herzegovina and their exploitation in traditional vs backslopping methods for kefir production was described in the chapter 3. Furthermore, the diversity in microbial dynamics, nutritional and volatilome profiles of traditional, commercial and backslopping methods was evaluated. This chapter is based on a paper submitted for publication on Food Research International.

The study of the potential role of essential oils as preservatives is presented in chapter 4. In this chapter the antifungal activity of seven different essential oils was evaluated against several yeast spoilage isolates belonging to different genera and collected from different food matrices. In particular, the attention has been focused on the possible use of these essential oils as preservatives against yeasts spoilage in yogurt. Moreover, the effectiveness of the antifungal activity of these essential oils was also tested against several isolates of *Zygosaccharomyces bailii* collected from filling creams of bakery products.

Chapter 5 describes the use of culture-dependent and culture-independent methods aimed to detect and quantify *Brettanomyces* spp. as spoilage agents of different Albanian wines. This chapter is based on a paper published on Journal of Food Science.

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Chapter 2 - Study of nicotinamide riboside production in craft beers

Introduction

Beer production

The beer production represents one of the oldest biotechnologies, in fact its origin can be placed around 7000 BC by Assyrian-Babylonian and Sumerians, as they were the first populations capable of producing a fermented cereal-based beverage (Axcell, 2007). This beverage subsequently reached Egypt and thanks to the Egyptians was known by the Greeks, Romans and Celts who contributed to a rapid spread throughout Europe.

Now, the term beer is used to indicate a beverage that derives from a biochemical process which consists of the transformation of the sugars within wort beer through the yeast action. This process is an alcoholic fermentation process that leads to the production of ethanol, carbon dioxide and other compounds, defined secondary, important in the characterization of the product (Cabras et al., 2004). The raw materials necessary for the beer production are water, barley (*Hordeum vulgare*), hop (*Humulus lupulus*), and yeast (genus *Saccharomyces*). Other cereals can be added in addition to barley, such as rice, wheat, corn, rye, oats, but in a percentage not exceeding 40%. Indeed, the barley must be always predominant (Stewart, 2016).

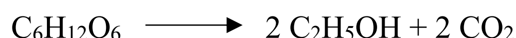
The brewing process can be divided in four different phases: malting (transformation of barley into malt), mashing (production of wort), fermentation (transformation of sugars in ethanol, carbon dioxide and secondary compounds) and downstream processes (such as maturing, bottling, packaging).

Barley and other cereals eventually present, must be subjected to the malting process which consists in seed germination by its hydration and in seed drying by heat treatment. This process allows to produce the hydrolytic enzymes needed for the conversion of starch into fermentable sugars.

The purpose of the mashing is to solubilize the largest possible quantity of substance originally insoluble in the malt and transform them by enzymatic reactions, favoured by appropriate temperatures during the process. At the end of this phase the wort is obtained (Rehberger et al., 1995; Kunze, 1996; Goldammer, 2000; Boulton et al., 2006). The first phase of the mashing consists of the grinding of the seeds in order to increase the contact surfaces with the water to facilitate the extraction of the substances contained in the malt and to favour the enzymatic reactions. The second phase consists in mixing the malt with water to solubilize the greatest possible quantity of substances through the action of enzymes and to heat this mixture up to the optimal temperatures for the enzymatic activity, after which, appropriate stops (at times and temperatures defined) are made to hydrolyse these substances. These enzymes convert the starches to dextrin (not fermentable) and to fermentable sugars such as maltose. After that, a filtration process (lautering) is performed which allows the separation of the solid part, the grain, from the liquid part, the wort. Now the wort is boiled for 45-90 minutes to sterilize it, which is essential for optimal fermentation by yeast. Moreover, during this process there is the addiction of hop, a fundamental ingredient which has a dual role: aromatic (it is responsible for the bitter taste) and bacteriostatic. At the end of the boil, solid particles in the hopped wort are separated out, in a vessel called whirlpool. After the whirlpool the wort must cooled by a heat exchanger to obtain the desired temperature to add yeast and start the fermentation.

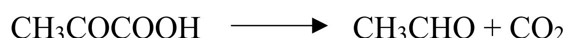
In the fermentation step, made by yeast, the sugars are converted into ethyl alcohol with the development of heat, carbon dioxide and many other so-called secondary products. This process is divided into two phases: primary fermentation and secondary fermentation (maturation). During the primary fermentation, of short duration and very vigorous, almost all the sugars are fermented, and

many secondary compounds of yeast metabolism are formed. The general equation of alcoholic fermentation is that indicated in the early XIX century by Gay-Lussac:



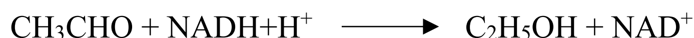
$$C_6H_{12}O_6 \longrightarrow 2\ C_2H_5OH + 2\ CO_2$$

Glucose demolition occurs through glycolysis that end with the production of pyruvic acid (Sols et al., 1971). Then, this molecule is decarboxylate to acetaldehyde by a pyruvate decarboxylase



$$CH_3COCOOH \longrightarrow CH_3CHO + CO_2$$

So, acetaldehyde is reduced to ethyl alcohol, with the contemporary reoxidation of NAD^+ by the action of an alcohol dehydrogenase



$$CH_3CHO + NADH{+}H^+ \longrightarrow C_2H_5OH + NAD^+$$

The yeast strain used for the brewing process belong to the genus *Saccharomyces* spp., and traditionally they are divided into yeast for low fermentation, namely *Saccharomyces pastorianus* (operating temperature 8-15°C) and yeast for high fermentation, namely *Saccharomyces. cerevisiae* (operating temperature 15-23°C). The use of *S. cerevisiae* cultures (top yeast) produces a high fermentation beer (top fermentation) called Ale, in which the yeast tends to rise to the surface and position in the foam. On the opposite *S. pastorianus* (bottom yeast) produces a low fermentation (bottom fermentation) in which the yeast at the end of the fermentation process are found on the bottom of the beer due to their ability to flocculate (Lager beer) (Speers et al., 1992; Iserentant, 1994; Verstrepen et al., 2003; Lodolo et al., 2008). Over 90% of the beers produced in the world are Lager beers, while the rest is represented by Ale beers and beers obtained from spontaneous fermentation, among which the most famous are the Belgian Lambic beers.

Beers can be further classified according to alcohol concentration starting from alcohol-free beer (AFB) at 0-0.05% (v/v). The concentration is defined by 'alcohol by volume' (ABV) in units of cm^3 ethanol/100 cm^3 beer or % (v/v). Most beers resides in the range of 3-6% (v/v), although higher gravity brewing can produce beer with alcohol content up to 10% (v/v) or more (Liguori et al., 2018).

At the end of this primary fermentation (about 7 days) most of the yeast is collected and separated from the beer (green beer) which undergoes a secondary fermentation (maturation) which ends the fermentation of the residual sugars. This maturation step, due to the yeast still present in the green beer, is long lasting, not very vigorous, and occur at lower temperature than primary fermentation (0-2°C for low fermentation and 7-10°C for high fermentation).

The beer, at the end of maturation (generally 3-4 weeks), must be subjected to filtration processes in order to separate the suspended solids and to pasteurization, finalized to have a more stable final product. In a few cases, there is another step of hop addiction to the beer. This step, called dry hopping, can be performed before the fermentation, at the end of fermentation, or during a second fermentation in the bottle.

Craft beers, unlike the industrial ones, are usually subjected to a second fermentation process in the bottle, by the addition of sugars and yeast. The beer produced in craft breweries differs from the industrial one also because they are consumed unfiltered and unpasteurized. This means that there are differences both in terms of organoleptic characteristics of the product and in terms of production technology. Moreover, craft breweries produce mainly Ale beers and so they utilize predominantly *S. cerevisiae* yeast strains.

Saccharomyces cerevisiae

S. cerevisiae is a yeast belonging to the Saccharomycetaceae family and it is one of the most studied eukaryotic model organisms in molecular and cell biology. It is the microorganism involved by ancient times in the most common type of fermentations such as winemaking, baking and brewing.
S. cerevisiae cells may present as single, paired or chained and their optimum temperature of growth is 32-33°C. *S. cerevisiae* cells can be globose or sub-globular, elliptical or cylindrical. Sometimes the cells can also be very long (over 30 µm compared to common 5-10 µm) and can give rise to

pseudohyphae, also branched. Cells normally divide by budding, producing a genetically identical daughter cell from a mother cell. When two haploid cells (which contain one copy of each chromosome) mate and fuse, they yield a diploid cell, which contains two of each chromosome. A diploid can either grow by budding or undergo meiosis. In this last case, the cell is transformed into an ascus containing 1 to 4 globose or elliptical haploid spores (Figure 1). Spores can be individually isolated and propagated as haploid spore clones or mated to one another to form a diploid. (Mell and Burgess, 2001; Kurtzman et al., 2011).

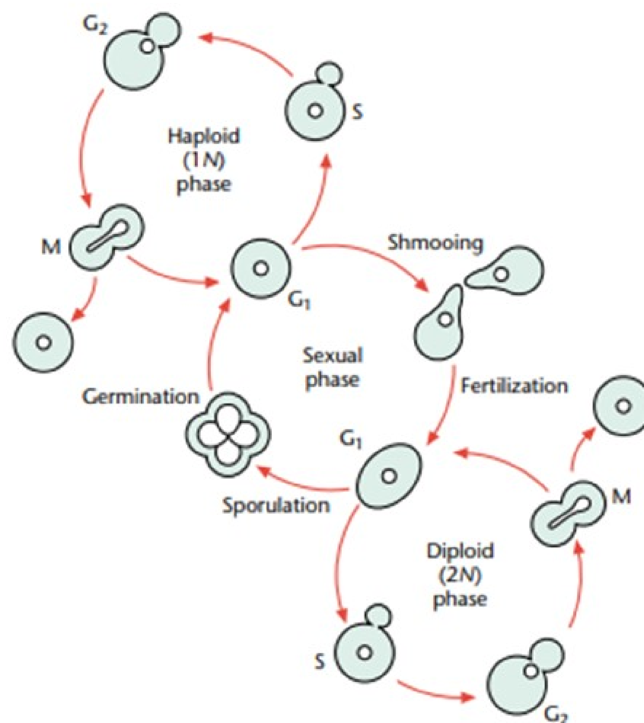


Figure 1. The life cycle of *S. cerevisiae* (Mell and Burgess, 2001).

Its use in brewing takes place thanks to some peculiar characteristics: high fermentation vigour, high alcohol content, high resistance to antiseptics, high adaptability to different conditions.

Moreover, *S. cerevisiae* produces the lowest overall quantities of secondary compounds and therefore gives rise to fermentations with the highest yield in ethyl alcohol.

Yeasts have two pathways for ATP production from glucose: respiration and fermentation. Both pathways start with glycolysis, which results in the production of two molecules of pyruvate and ATP for glucose. In fermentation, pyruvate is then turned into ethanol. This process does not produce additional ATP but recycles the NAD^+ consumed in glycolysis and thereby provides a way of oxygen-independent ATP production. In respiration, pyruvate is completely oxidized to CO_2 through the tricarboxylic acid cycle (TCA cycle) and oxidative phosphorylation (OXPHOS), which yields additional ATP but requires oxygen (Figure 2). In the presence of oxygen and low sugar concentrations, *S. cerevisiae* uses glucose which induces high biomass production and low ethanol production. At high sugar concentrations (as in wort), *S. cerevisiae* produces a large amount of ethanol because it metabolizes glucose only by fermentation which shows a high rate of sugar utilization. In this way, even in the presence of oxygen, respiration is repressed and therefore biomass production is limited (Crabtree effect) (Crabtree, 1929; De Deken, 1966).

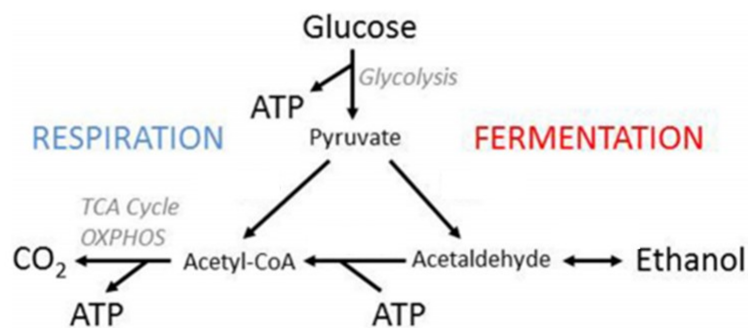


Figure 2. Yeast energy metabolism (Pfeiffer and Morley, 2014 modified).

Vitamin B_3

Since, it is known that *S. cerevisiae* is able to actively secrete Nicotinamide Riboside (NR), a form of vitamin B_3 (Bogan et al., 2009; Lu et al., 2009), its presence in fermented foods and beverages, including beer, has been hypothesized (Chi and Sauve, 2013).

The vitamin B_3, also called Niacin or vitamin PP (Pellagra-Preventing), includes three similar organic molecules: Nicotinic Acid (NA), its amide Nicotinamide (Nam), and Nicotinamide Riboside (NR) (Figure 3) a new recently discovered form of this vitamin (Bieganowski and Brenner, 2004).

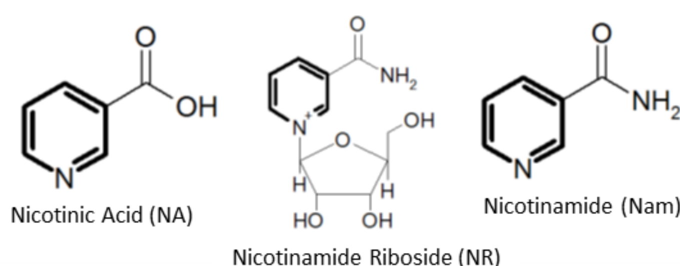


Figure 3. Molecular structure of Nicotinic Acid (NA), Nicotinamide Riboside (NR), Nicotinamide (Nam).

The discovery of the niacin as vitamin comes from the research on the causes of the plague known as pellagra, a disease whose classic symptoms are diarrhoea, dermatitis, dementia, death ("the four Ds"). Pellagra was initially considered an infectious disease, until 1914, when Goldberger verified the hypothesis that the disease could be caused by a food deficiency and discovered that replacing a wheat-based diet with eggs, milk and meat, was an excellent strategy for treatment and prevention (Goldberger and Willets, 1914). Subsequent studies demonstrated that NA and Nam were able to cure the "black tongue", a dog disease considered equivalent to the human pellagra (Elvehjem et al., 1937). Moreover, it was reported that these sick dogs had very low levels of NAD^+ and $NADP^+$ (Figure 4) in liver and muscles that could be increased by the administration of NA and Nam. Therefore, it was concluded that NA and Nam were precursors of NAD^+ and $NADP^+$ coenzymes and NA and Nam were essential for the treatment of human pellagra (Axerold et al., 1939). Furthermore, after its discovery, NR was also considered a precursor of NAD^+ and $NADP^+$.

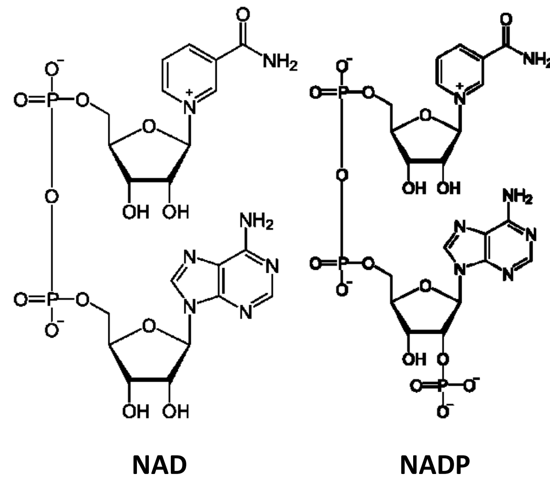


Figure 4. Molecular structure of NAD^+ and $NADP^+$.

Figure 5 shows that NA, Nam and NR taken with the diet are transported inside the cells where they can be transformed into NAD^+. Moreover, Nam and NR can be also formed in the extracellular space from dietary NAD^+ and nicotinamide mononucleotide (NMN) by a combined action of enzymes of the intestinal mucosa and of the gut microbiota (Bogan and Brenner, 2008). Likewise, Nam can be deamidated to NA (Figure 5).

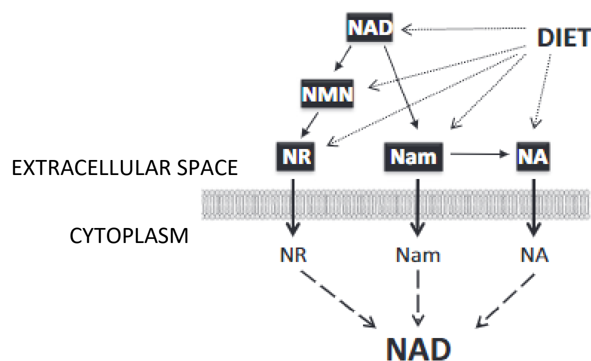


Figure5. Scheme of dietary pyridines uptake for intracellular NAD^+ biosynthesis in humans (Ummarino et al., 2017).

Therefore, NA, NR and Nam are dietary precursors of NAD^+, but at the same time NAD^+ itself and its derivative NMN can be precursors of the vitamins.

Metabolism of NAD^+ in yeasts

In yeast, NAD^+ is generated by *de novo* synthesis and by salvaging nicotinic acid (NA), nicotinamide (Nam) and nicotinamide riboside (NR), the three salvageable NAD^+ precursor vitamins (Figure 6) (Belenky et al., 2011). The *de novo* pathway is initiated by six different enzymes that produce nicotinic acid mononucleotide (NaMN) from tryptophan. NaMN is then converted to NAD^+ by enzymes that are also involved in the NA → NAD^+ conversion. In fact NA, which is imported by the transporter Tna1, is converted to NAD^+ by the 3-step Preiss-Handler pathway (Preiss and Handler, 1958; Lorente et Dujon, 2000). In the first step, NA is converted to NaMN by the nicotinate phosphoribosyltransferase Npt1; NaMN is then converted to nicotinic acid adenine dinucleotide (NaAD) by nicotinamide adenylyltransferases (Nma1/2) and finally NaAD is amidated to NAD^+ by the enzyme NAD synthetase (Qns1) (Preiss and Handler, 1958). Furthermore, NA can be released by a transporter different from Tna1. Nam, the transporter of which is unknown, is both of dietary origin and is produced by the intracellular NAD^+ consuming reactions. Nam is converted to NA by nicotinamidase Pnc1, for entry into the Preiss-Handler pathway (Ghislain et al., 2002). NR is imported by the transporter Nrt1, and converted into NAD^+ by two distinct pathways. The first pathway utilizes the NR kinase Nrk1, to produce NMN, which is then converted into NAD^+ by Nma1/2 (Bieganowski and Brenner, 2004). The second pathway uses three nucleosidases to cleave NR into Nam, which is then converted to NAD^+ as described above (Belenky et al., 2007; Tempel et al., 2007; Belenky et al., 2009). Moreover, *S. cerevisiae* can release NR through a Nrt1-independent mechanism, even if the purpose of this export is unknown.

Nicotinic acid riboside (NAR) is an intracellular metabolite that can be also utilized as a NAD^+ precursor (Lu et al., 2009). Indeed, NAR is a substrate of two enzymes that can convert it into NaMN and NA, respectively (Tempel et al., 2007). In addition, there are two enzymes that are capable to convert NMN and NaMN to NR and NAR respectively (Bogan et al., 2009).

Thus, these data indicate that NAD+ metabolism has an extracellular component that works in conjunction with intracellular metabolic pathways to regulate intracellular NAD+ precursor levels, and store vitamins extracellularly.

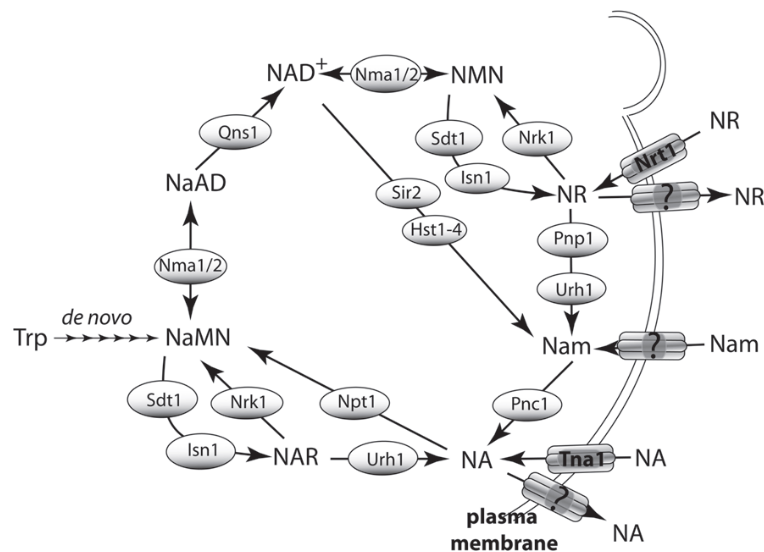


Figure 6. NAD+ biosynthesis in yeasts (Belenky et al., 2011).

Functional role of NAD+, the active biological form of vitamin B_3

NAD+ as a redox coenzyme

NAD+ and its oxidised and/or phosphorylate derivatives NADP+, NADH and NADPH are essential cofactors for oxidoreductase enzymes, and therefore for the energy metabolism of the cell. NAD+ and NADP+ are used as redox coenzymes in both anabolism (such as gluconeogenesis, synthesis of fatty acids, synthesis of nucleotides) and in catabolism (such as glycolysis, catabolism of fatty acids). Furthermore, NADH delivers its electrons to its final acceptor (O_2) through the transporters that form the mitochondrial respiratory chain. Approximately 100 human enzymes utilize NAD+ as a cofactor

for oxidation/reduction reactions catalysed by dehydrogenases or oxidoreductases and a similar number utilizes $NADP^+$ (Placzek et al., 2017).

NAD^+ as substrate for enzymes

NAD^+ is also used by many classes of enzymes (such as sirtuins, PARPs, CD38) that use it as a consumable substrate in several reactions (Belenky et al., 2007).

CD38 is a transmembrane glycohydrolase that cleave NAD^+ to generate Nam and adenosine diphosphoribose (ADPR). To a lesser extent, it can also act as an ADP-ribosyl cyclase that catalyse the hydrolysis of NAD^+ to generate Nam and cyclic ADPR (cADPR). Both ADPR and cADPR are secondary intracellular messengers used in the mobilization and release of Ca^{2+} ions in many cell types (Berthelier et al., 1998; Malavasi et al., 2008; Wei at el., 2014). CD38 serves also as an antigen and ligation by antibodies can trigger a wide range of responses in various types of cells. It is ubiquitously expressed and has been implicated in energy metabolism, cell adhesion and various aspects of the immune response. It has been also linked to human diseases such as Parkinson's, ovarian cancer and leukaemia (Quarona et al., 2013).

PARPs proteins, which includes both poly-ADP-ribose-polymerases and mono-ADP-ribose-transferases are NAD^+-responsive signalling proteins. PARPs cleave NAD^+ into Nam and ADP-ribose, simultaneously transferring the ADP-ribose moiety to asparagine, aspartic acid, glutamic acid, arginine, lysine and cysteine residues on target proteins, forming branched poly-ADP-ribose polymers, thus modifying the biological functions of proteins (Bai, 2015). PARPs are involved in a wide variety of cellular functions such as DNA repair, mitosis, organization of the nuclear envelope, post transcriptional regulation of mRNA, regulation of circadian rhythm and regulation of transcription of genes required for immune and inflammatory response. (Rajman et al., 2018).

Another family of proteins that uses NAD^+ as a substrate to modify target proteins are sirtuins. These enzymes regulate a wide variety of proteins involved in processes that include mitochondrial metabolism, inflammation, DNA repair, cellular communication, meiosis, autophagy, circadian rhythms and apoptosis (Haigis and Sinclair, 2010). The main reaction is the removal of an acetyl group from lysines on target proteins (deacetylation). Since the enzymatic reaction catalysed by sirtuins requires NAD^+, whose concentration is determined by the nutritional status of the cell, the expression and activity of these enzymes is coupled to changes in cellular energy/redox status. Therefore, sirtuins can be considered metabolic and stress sensor proteins (Gomes et al., 2015).

Effects of vitamin B_3 on human health

It is known that NAD^+ is in a constant state of synthesis, degradation and recycling in cytoplasm and within organelles such as nucleus, Golgi and peroxisomes (Anderson et al., 2003). Indeed, there are rapid, local fluctuations of NAD^+ levels (Zhang et al., 2012). The concentration and the distribution of NAD^+ and its metabolites are different depending on the cell compartment and change in response to physiological stimuli and cellular stress. Moreover, studies suggest that NAD^+ levels decline with age at a system level in diverse organisms, including humans, contributing to the development of many age-associated pathophysiologies (Canto et al.2015; Imai and Guarente, 2014; Verdin, 2015).

Although B_3 deficiency is a rare event in industrialized nations (Graham, 1993), diseases such as neurodegenerative diseases (Parkinson's, Alzheimer's), metabolic syndrome (e.g. Hartnup's disease), HIV, autoimmune diseases, alcohol dependence, anorexia and diseases related to aging seem to reproduce the symptoms of pellagra. These symptoms can be treated in several cases by administering vitamin B_3 (Penberthy, 2007). Indeed, NA is a hypolipidemic drug, while Nam treatment can have beneficial effects against obesity and type-2 diabetes (Yang et al., 2004). However, the clinic use of NA can induce cutaneous flushing, while Nam can cause hepatotoxicity (Kang-Lee et al., 1983).

Therefore, much interest is currently devoted to the potential therapeutic value of NR and NMN supplementation as NAD^+ precursors.

Health effects of NR

Protection against metabolic and mitochondrial disorders

Several studies have shown that the administration of NR is able to prevent or treat diseases related to aging and metabolic disorders (Yang and Sauve, 2006; Yoshino et al., 2018).

To evaluate the effects of NR in living mammals, mice treated with NR (400 mg /Kg /day), after a high-fat-diet-feeding, showed an increase in NAD^+ levels in muscle and liver that leads to activation of SIRT1 and SIRT3. SIRT1 activates transcriptional factors that lead to an increase in fatty acid oxidation and mitochondrial biogenesis, while SIRT3 in mitochondria stimulates the expression of specific target genes that give protection against oxidative damage (Canto et al, 2012). Therefore, these mice were protected from weight gain, were more insulin sensitive, and had increased mitochondrial content in skeletal muscle and brown adipose tissue compared with untreated controls Moreover, it has been demonstrated that NR has a greater capacity to increase the level of NAD^+ in mitochondria compared with other NAD^+ precursors such as NMN, Nam and NA (Canto et al., 2012). Thus, NR through the increase of mitochondrial NAD^+ could provide several potential benefits in disease states in which cell death and reactive oxygen detoxification are abnormal. These benefits are mediated by the action of SIRT3. Indeed, SIRT3 represses the formation of the mitochondrial permeability pore and stimulates the reactive oxygen species detoxifying enzymes such as superoxide dismutase 2 (Chen et al., 2011). Another study has shown that the intake of NR in mice affected by mitochondrial myopathy, induces an increase in mitochondrial biogenesis, measured by the increased content of cristae in muscle tissue (Khan et al., 2014). All these data suggest that NR could be an

agent for the treatment of metabolic disorders and reactive oxygen syndromes associated with mitochondrial dysfunction.

Neuroprotective effects

Several researches have been devoted to the study of the effects of NR in treatment of neurodegenerative diseases, such as Alzheimer's disease. This pathology involves the formation of amyloid plaques in the dendritic area and on the surface of neuronal cells of the cerebral cortex, which hinder neurotransmission. Alzheimer's patients develop progressive memory loss over time and loss of cognitive skills that leads to dementia. It has been shown that the administration of NR in a mouse model of Alzheimer induces an increase in the amount of NAD^+ in neuronal cells, that leads to a greater cognitive capacity of the animals. This effect is related to an increase in SIRT1 activity on peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1α). This transcription factor is essential for the regulation of the formation and accumulation of Aβ-amyloid plaques. Indeed, PGC-1α induces the expression of the BACE enzyme that degrades the amyloid plaques in polypeptide fragments with lower molecular weight (Amyloid Precursors Protein) (Gong et al., 2013).

NR has been shown to prevent noise-induced hearing loss. In fact, the increasing of NAD^+ amount and consequently of SIRT3 activity by NR administration, preserves the integrity of synaptic contacts which can be broken or degenerated by a persistent exposure to noise (Brown et al., 2014).

There are several additional studies that illustrate the neuroprotective value of NAD^+ in brain and the ability of NR to increase NAD^+ level in this tissue. Therefore, NR could have clinical relevance in future neurotherapy approaches.

Effects on cellular longevity

In yeast, assimilation of endogenous NR has been shown to be essential for caloric restriction-mediated life span extension. (Lu et al., 2009; Haigis and Guarente, 2006). In fact, the experiment on *S. cerevisiae* showed that the presence of NR in culture medium led to an increase in the amount of intracellular NAD^+ and to a greater Sir2 (homologue of human SIRT2) activity which is related to the increase of the lifespan. Moreover, it has been shown a small but significant extension of lifespan in mice treated with NR (Zhang et al., 2016). Experiments on the effects of NR on muscle stem cells (MuSCs), neural stem/progenitor cells (NSPCs), mesenchymal stem cells (MSCs) (Ryall et al., 2015; Stein and Imai, 2014; Quinn et al., 2013), show the anti-aging properties of this vitamin which is now considered a supplement food with the potential to extend the life span of living beings.

Other effects

There several studies that shows as NR improves liver health in a variety of contexts. It can reduce fat accumulation, inflammation, and the development of fibrosis (Lee et al., 2015; Gariani et al., 2016; Zhou et al., 2016; Gomes et al., 2016). In the regenerating liver (following partial hepatectomy), NR reduces lipid accumulation, promotes hepatocyte replication, increases hepatic ATP content, and leads to faster regain of liver weight (Mukherjee et al., 2017). All these observations suggest that enhancing NAD^+ content by NR is a promising therapeutic strategy to improve liver health.

NR has been shown to have beneficial effects also in muscle disorders. For example, administration of NR can improve stem cell function and partially ameliorate the muscle wasting phenotype in a Duchenne's muscular dystrophy mouse model, leading to hope for a human therapy application (Ryu et al., 2016; Zhang et al., 2016).

Health effects of NMN

Administration of NMN (Figure 7) to mice leads to an enhance of NAD^+ biosynthesis in several tissues, such as pancreas, liver, adipose tissue, heart, kidney, skeletal muscle under normal and pathophysiological conditions (Yoshino et al., 2018).

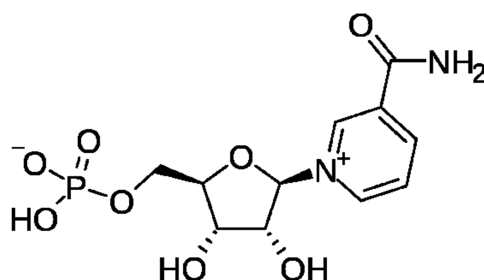


Figure 7. Molecular structure of NMN

Since it has been demonstrated that a long-term oral administration of NMN in mice is safe and well tolerated and does not cause any deleterious or toxic effect, it could offer broad applications and therapeutic potential. Indeed, there are several studies that show the beneficial effects of NMN in various diseases.

It has been demonstrated that pancreatic β cells are very sensitive to NMN administration. Indeed, an injection of 500 mg/kg of NMN enhances insulin secretion in diabetic mice (Caton et al.,2011; Yoshino et al., 2011). NMN enhances also the insulin action, because it ameliorates hepatic insulin resistance by increasing NAD^+ biosynthesis and SIRT1 activity, hence modulating the expression of genes related to inflammation, oxidative stress, and circadian rhythms (Yoshino et al., 2011). NMN administration plays a role also in adipose tissue by increasing NAD^+ biosynthesis. Indeed, it can suppress the age-associated adipose tissue inflammation and can improve whole-body insulin sensitivity (Mills et al., 2016). These studies suggest that the increase of NAD^+ in adipose tissue could

be of therapeutic value to combat insulin resistance, which is an important risk factor of type 2 diabetes and cardiovascular disease.

Other studies have demonstrated that NMN can improve several neuronal functions in the brain. NMN administration improves cognition and memory in Alzheimer's mouse model (Wang et al.,2016; Yao et al., 2016), and protects neurons from cell death after ischemia or intracerebral haemorrhage (Park et al., 2016; Wei et al., 2017).

In addition to anti-diabetic and neuroprotective effects, NMN administration also determines benefits in other tissues such as kidney, heart, liver and eyes (Yoshino et al., 2018).

Interestingly, different studies have shown that NAD^+ replenishment by NMN administration is more effective in aged mice and suppresses age-associated physiological decline (Mills et al., 2016). These data suggest the importance to evaluate the effects of NMN administration on other age-associated diseases such as cancer and sarcopenia, and aging and lifespan in mice.

Object of work

The are many evidences that administration of the vitamin NR and its precursor NMN causes a significant increase in the intracellular content of NAD^+ in many murine tissues and organs, which is reflected in an improvement of energy metabolism and mitochondrial functions. Therefore, dietary supplementation of NR and/or NMN is now considered a promising means for the treatment of metabolic, mitochondrial and neurodegenerative disorders. Considering the beneficial effects attributed to these molecules, it is important to understand their distribution in foods. NMN has been detected in many natural foods such as broccoli, tomatoes, mushrooms, cabbage, shrimp, avocado and beef meat with a maximum concentration of 1.88 mg/100g of food (Mills et al., 2016), while NR presence has been documented at micromolar concentrations only in milk (Bieganowski and Brenner, 2004; Ummarino et al., 2017). However, considering the ability of *S. cerevisiae* to release NR in a culture medium (Lu et al., 2009), the presence of this vitamin has been hypothesized in fermented foods and beverages, including beer (Yang and Sauve, 2006). Therefore, the aim of this work was to detect and quantify the amounts of NR and its precursors NMN and NAD^+ in different craft beers by using an fluorometric assay. On the basis of results, the successive laboratory-scale beer productions have focused the attention on the role of hop, of wort and of different *S. cerevisiae* strains, in NR production, with the aim of increase its level. Considering the benefits related to beer consumption (Bamforth, 2002; Sohrabvandi et al., 2012) and the knowledge about many methods for low alcohol beer production (Brányik et al., 2012), this one could be the first step to produce a low alcohol beer with a high vitamin B_3 content.

Materials and methods

Craft beer sampling

Ten craft beers of different brew styles were analysed with the aim of detecting and quantifying NAD^+, NR and NMN. In particular, two samples for each beer from different batches were collected from two different craft breweries in the province of Ancona (Marche region). The samples 12, G1, G2, G3 and G4 contained wheat as additional ingredient, while oat flakes are added as additional ingredient in G5. Instead G6 contained both wheat and oat flakes as additional ingredients. All the worts used to produce these beers were collected during the boiling and stored at 4°C. The yeast strains used by the breweries (four strains of *S. cerevisiae* and one of *S. pastorianus*) (Fermentis) were obtained and stored at 4°C. *S. cerevisiae* strains were: S-33; US-05; S-04; WB-06. The *S. pastorianus* strain was W34/70. Figure 8 shows a flow diagram of manufacture of the craft beers under study.

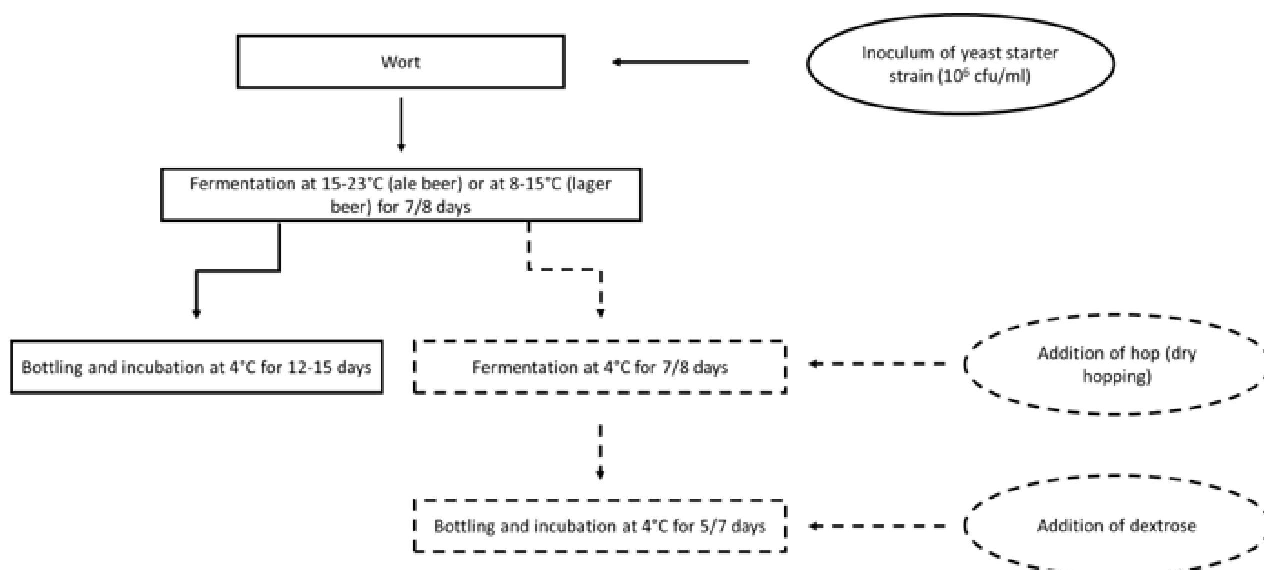


Figure 8. Flow diagram of manufacture of a craft beer. Dashed lines indicate optional steps for G5 and G6 beers

Fluorometric assay for NR, NMN and NAD^+ quantitation

Principle of the assay

Figure 9 shows the principle of the fluorometric assay. Briefly, it enables quantification of NR and NMN by means of their stoichiometric conversion to NAD^+, which is measured by a fluorometric cycling assay described in (Graeff and Lee, 2002). NR is phosphorylated to NMN by NR kinase (NadR), and the formed NMN is adenylylated to NAD^+ by NMN adenylyltransferase (NMNAT). The produced NAD^+ is cycled by the combined action of alcohol dehydrogenase (ADH), that reduces NAD^+ to NADH, and diaphorase that re-oxidizes NADH to NAD^+. Simultaneously to these reactions, there is conversion of resazurin into resorufin, which is highly fluorescent. The rate of fluorescent resorufin production is proportional to the amount of NAD^+, hence to NMN or NR present in the analysed sample.

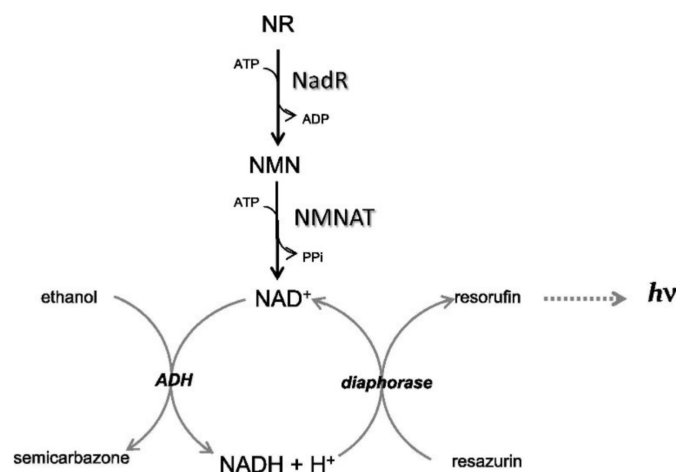


Figure 9. Principle of the coupled assay (Ummarino et al., 2017).

Preparation of samples

All the beer samples and all of the wort samples were subjected to acid-soluble nucleotides extraction. To this end, 1 ml of sample was centrifuged at 16000 x g for 5 minutes at room temperature. Nucleotides were extracted adding 250 µl of 1.2 M $HClO_4$ to 500 µl of sample supernatants. After 15 minutes at 4°C, samples were centrifuged at 16000 x g for 5 minutes at room temperature. 700 µl of the supernatants were added to 170 µl of 1.0 M K_2CO_3 to reach a pH value of about 7.0. Neutralized samples were incubated at -20°C for 20 minutes, and they were centrifuged at 16000 x g for 5 minutes at room temperature. The supernatants to be used for the fluorometric assay were stored at -20°C.

Expression and purification of the ancillary enzymes NadR and NMNAT

Expression and purification of NadR was performed as previously described (Ummarino et al., 2017). The NadR activity was measured spectrophotometrically through the NMNAT-coupled assay described in (Ummarino et al., 2017). Expression and purification of mouse NMNAT (isoform NMNAT3) was performed as described in (Orsomando et al., 2012) with the following modification: active fractions eluted from the affinity column were pooled and desalted on a PD-10 column equilibrated in 50 mM HEPES/KOH, pH 7.5, 20% glycerol. The enzymatic activity was assayed by the spectrophotometric assay described in (Balducci et al., 1995).

Analysis of samples

Seven different assay mixtures were prepared for the simultaneous determination of the three analytes in each sample. All mixtures were prepared as described in (Ummarino et al., 2017). After 20 min incubation at 37 °C, the NAD^+ formed in each mixture was subjected to the cycling reaction and the

fluorescence increase was recorded as described in Zamporlini et al. (2014). Both the preparation of the cycling reagents and the monitoring of the fluorescence increase were performed as described in Zamporlini et al. (2014). The preparation of the NR standard curve and the calculations for the quantification of the three molecules were performed as described in Ummarino et al. (2017). All the assay's steps were carried out in the wells of microtiter plates. In this way, it was possible to record the fluorescence increase in all samples simultaneously.

Fermentation trials

NR, NMN and NAD^+ determination in lab-scale produced beer

Based on the results of the screening of the beers the G5 beer has been chosen for further studies. In detail, the yeast strain US-05 was grown on Yeast Extract Peptone D-glucose (YPD) (yeast extract 10 g L^{-1}, peptone 20 g L^{-1}, D-glucose 20 g L^{-1}) medium at 25°C for 72 hours. It was inoculated in a sterile flask (250 ml) containing 100 ml of G5 wort added with hop to reach a final concentration of about 6 Log cfu mL^{-1}. The fermentation process took place for 8 days at 21°C (at the end of this time has been obtain the green beer). At the 8th day of fermentation has been added two different hops (dry hopping), in pellet form, provided by the brewery and stored at 4°C. These hops were amarillo (alpha acid: 9.0%) (4 g L^{-1}) and centennial (alpha acid: 8.5%) (4 g L^{-1}). After this addition the fermentation continued at 4°C for 7 days (15 days from the start of the fermentation). At the 15th day of fermentation has been added dextrose (7 g L^{-1}) dissolved in 600 µl of sterile water after heating at 100°C for 5 minutes. Then, the brewing process continued at 4°C until the 22nd day (the minimum time of fermentation for the brewery before obtaining a saleable final product).

At time 0 (at the moment of inoculum) and during fermentations, at different days from inoculum (2-3-5-7-8-9-10-11-14-15-16-17-18-21-22), 2 mL of medium (either experimental G5 laboratory beer

or YPD) was removed and a total of 16 aliquots was collected. From each 2 mL aliquots: i) 1 mL was used for the yeasts enumeration on YPD agar (agar 18 g L^{-1}), following decimal serial dilutions on sterile peptone water (peptone 1 g L^{-1}); ii) 1 mL was used for the determination of the content of NR, NMN and NAD^+ through the fluorometric assay, as described in paragraph "Analysis of samples".

NR, NMN and NAD^+ determination in a lab-scale produced beer without hop addiction

To understand the effect of hop in the brewing process, two different fermentation trials have been set up: a G5 lab-scale produced beer (MB) and a G5 lab-scale control beer (M1). The yeast strain (US-05) has been inoculated in the lyophilized form in two different sterile flasks (250 ml), both containing 100 ml of G5 wort, to reach a final concentration of about 6 Log cfu mL^{-1}. MB fermentation was carried out as described above, while in M1 no hops and dextrose were added. These fermentations were prolonged up to 45 days to understand if the amount of NR continues to increase also after the 22nd day. At time 0 (at the moment of inoculum) and during fermentations, at different days from inoculum (1-2-3-4-7-8-9-14-16-18-21-23-28-31-35-45), 3 mL of experimental G5 laboratory beer was removed and a total of 17 aliquots was collected. From each 3 mL aliquots: i) 1 mL was used for the yeasts enumeration on YPD agar (agar 18 g L^{-1}), following decimal serial dilutions on sterile peptone water (peptone 1 g L^{-1}); ii) 1 mL was used for the determination of the content of NR, NMN and NAD^+ through the fluorometric assay, as described in paragraph "Analysis of samples"; iii) 1 mL was used for the determination of metabolites and enzymatic activities through HPLC analysis as described in paragraph "HPLC assay for the determination of NAD^+-consuming activities".

NR, NMN and NAD^+ determination in YPD inoculated with different *S. cerevisiae* strains

Two fermentations trials were setup inoculating *S. cerevisiae* US-05 and *S. cerevisiae* CBS 1171 (from the Centraalbureau voor Schimmelcultures, Filamentous fungi and Yeast Collection, The Netherlands) in two different flasks (1 L) containing 200 mL of YPD instead of wort. These strains were grown on YPD medium at 25°C for 72 hours. In parallel, another flask containing 200 ml of YPD without yeast was set up as a control. The amounts of cells inoculated were about 6 Log cfu mL^{-1}. The flasks were incubated for 9 days at 21°C. On that day each flask was split in two flasks and in one of them hop was added. All flasks were then incubated at 4°C until the 21st day of fermentation. This time dextrose was not added because on the base of previous trials it was not found to interfere on the production of NR. During the fermentations process 2 mL aliquots at different days from inoculum (4-6-9-11-13-16-18-21) were removed. 1 mL from each aliquot was subjected to fluorometric assay for the analysis of the molecules of interest as described in paragraph "Analysis of samples". 1 mL from each aliquot was used for the enumeration of yeasts during the fermentation that was monitored by viable counts on YPD agar.

HPLC assay for the determination of NAD^+-consuming activities

1 ml of medium (see paragraph "NR, NMN and NAD^+ determination in a lab-scale produced beer without hop addiction") was removed at different times during the fermentation, and it was centrifuged at 16000 x g for 5 minutes, at room temperature. The supernatants were collected and stored at -20°C. For the determination of NAD^+ consuming activities, incubation mixtures were prepared consisting of 50 mM sodium acetate, pH 4.2, 100 µM NAD^+ and 100 µl of the supernatants (additionally centrifuged after thawing, at 16000 x g for 5 minutes), in a final volume of 200 µl. Two control mixtures for each tested sample were prepared containing either buffer and sample without

NAD^+, or buffer and NAD^+ without the sample. At different times of incubation (3-24-48 h) at 20°C, 60 µl-aliquots of each mixture were added with 30 µl of 1.2 M $HClO_4$ to stop the reaction. After 15 minutes at 4°C the samples were centrifuged and 70 µl of supernatants were removed. 16 µl of 1.0 M K_2CO_3 was added to each supernatant to reach a pH of about 6.0. After centrifugation, 70 µl of each supernatant was injected into a Supelcosil LC18 DB column (5 µm, 250 x 4,6 mm), eluted as described in (Mori et al., 2014). Column temperature was maintained at 8°C.

Results and discussions

NAD^+, NMN, NR determination in craft beers

All the beer samples were treated as described in paragraph "Preparation of samples" and analysed for the presence of NR, NMN and NAD^+ through the fluorometric assay described in paragraph "Analysis of samples". The results are shown in Table 1.

SAMPLE (Alcohol by volume)	YEAST	NR [nmol/ml]	NMN [nmol/ml]	NAD^+ [nmol/ml]
10 (6.3 % vol)	**S-04** *S. cerevisiae*	ND ND	ND ND	10.90 4.98
11 (5.5 % vol)	**S-33** *S. cerevisiae*	ND ND	ND ND	3.24 4.78
12 (6.3 % vol)	**WB-06** *S. cerevisiae*	ND ND	ND ND	1.19 1.94
13 (5.4 % vol)	**US-05** *S. cerevisiae*	1.14 0.48	ND ND	3.57 3.07
G1 (6.0 % vol)	**W34/70** *S. pastorianus*	ND ND	ND ND	ND ND
G2 (6.6 % vol)	**S-04** *S. cerevisiae*	ND ND	ND ND	7.81 17.80
G3 (6.6 % vol)	**US-05** *S. cerevisiae*	1.27 0.76	0.75 1.09	4.58 4.67
G4 (5.8 % vol)	**WB-06** *S. cerevisiae*	ND ND	ND ND	1.39 ND
G5 (5.5 % vol)	**US-05** *S. cerevisiae*	1.92 3.25	ND 0.81	2.50 2.44
G6 (5.5 % vol)	**US-05** *S. cerevisiae*	2.91 2.71	ND 0.95	1.10 1.97

Table 1. NR, NMN, NAD^+ concentrations in different beers. Two samples for each beer were analysed. ND=Not Detectable

With the exception of G1, NAD^+ was present in all samples, at variable amounts, ranging from 1.10 nmol/ml (G6) to 17.80 nmol/ml (G2). Four samples (10, 11, G2, G3) contained more NAD^+ than human milk (3.6 nmol/ml Ummarino et al., 2017). Notably, the two samples that contained the highest amount of NAD^+ (10 and G2) were produced by using the same yeast strain of *S. cerevisiae* (S-04). This suggests that this strain has the capacity to produce and release high amount of NAD^+ during the brewing process.

NR was detected in four different samples (13, G3, G5, G6) while NMN was detected only in three ones (G3, G5, G6) with amounts generally lower than NR. The NR concentrations were generally slightly higher than those determined in bovine and human milk (1.7 nmol/ml and 1.3 nmol/ml respectively), but lower than in buffalo and donkey's milk (4.2 nmol/ml and 3.3 nmol/ml respectively) (Ummarino et al., 2017). NMN amounts were lower than those measured in many natural foods such as tomato, avocado and beef meat (from 0.78 nmol/mg to 4.79 nmol/mg) (Mills et al., 2016). Interestingly, beers containing NR and NMN were prepared with the same yeast strain (US-05).

All the beers analysed were prepared with a *S. cerevisiae* species, except for G1 which was produced by *S. pastorianus.* Notably, this beer was the only one that was negative for all the three molecules under study (NR, MN, NAD^+), thus suggesting that *S. cerevisiae* could be the *Saccharomyces* species involved in the secretion of NR as previously proposed by Belenky et al. (2011) and Chi and Sauve (2013) and also of NMN and NAD^+. Moreover, the NR and NMN secretion seemed to be strain-dependent since only the beers produced with *S. cerevisiae* starter strain US-05 showed the presence of both these metabolites.

All the worts with hop (ready for yeast inoculum) were analysed and were found negative for the presence of NR and its metabolic precursors (not shown).

Prompted by these results it was decided to make a replicate of the sample G5 on a laboratory scale (G5L), to monitor the production of NR, NMN and NAD^+ during all the fermentation process. The

choice of G5 sample was made based on the amounts of the three molecules in this beer (G5 had the second highest concentration of NR) and the availability of the wort by the brewery.

NR, NMN and NAD^+ determination in lab-scale produced beer

The results of the monitoring of the production of the three metabolites under study along the fermentation process of G5L beer obtained with the fluorometric assay for are shown in Figure 10.

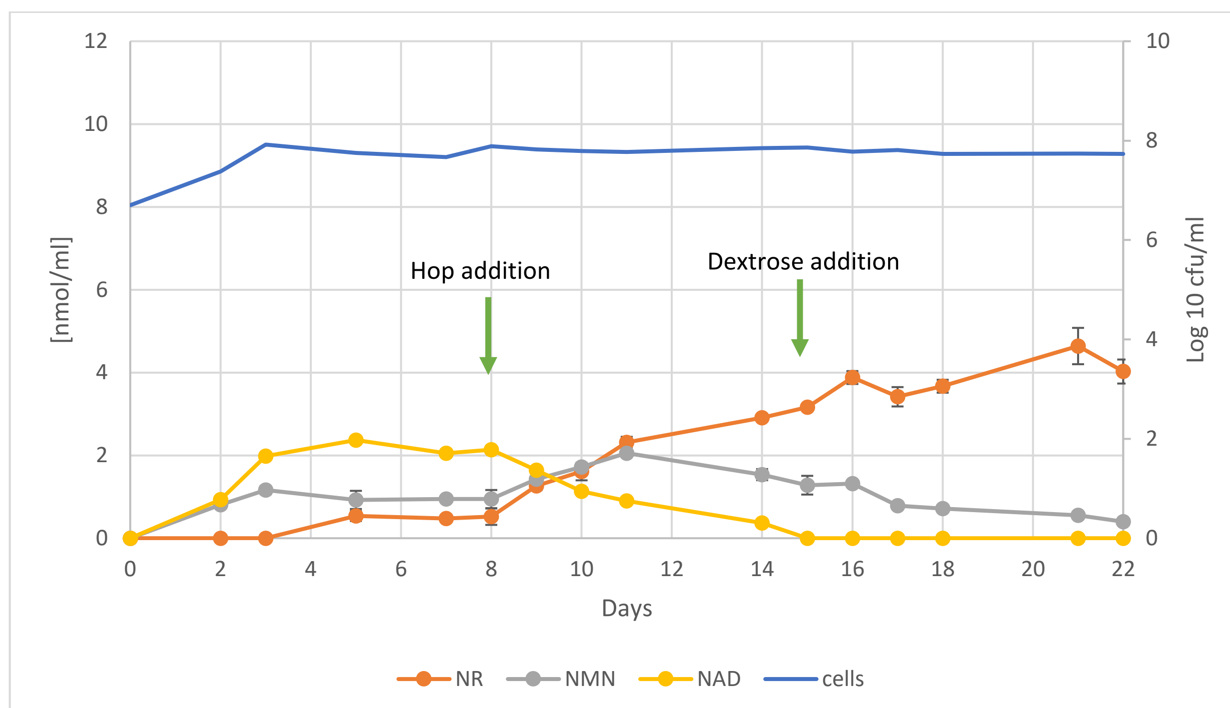


Figure 10. Concentrations of NR, NMN, NAD^+ and viable cells in G5L during the fermentation process.

At the 2nd day of fermentation the concentration of the yeast strain reached the value of 7.5 Log cfu mL^{-1} which remained constant until the end of the process.

As shown in Figure 10, the amount of NR increased during all the brewing process, with a burst after the addition of hop at the 8th day. In fact, the value of NR shifted from 0.52 nmol/ml at the 8th day to

1.27 nmol/ml at the 9th day, underlining an increase over the double. Instead, the addition of sugar did not provide a change of this trend. The trend of NAD^+ was very different from that of NR: at the beginning there was an increase of the amount, (maximum level at the 5th day), but after the 9th day there was a decrease which led to a value under the detection limit of the assay already at the 15th day of the fermentation process. Altogether, these results suggest that the addition of the hop might be responsible for the increase of NR and the decrease of NAD^+.

NMN amount was constant up to the 9th day, after that there was an increase that lead to a peak at the 11th day (2.05 nmol/ml), but from that moment on there was a progressive reduction and the amount was close to the detection limit in the last day of the fermentation process.

By comparing the levels of the molecules of interest in the beer and in the lab-scale beer, was found that the final amount of NR in G5L beer was much higher to that in G5 beer, and NAD^+ was absent in the beer produced in the laboratory. Moreover, NMN amount in the lab-scale beer was close to the limit of detection of the assay and it was therefore comparable with that measured in the craft beer.

The differences in the amount of NAD^+ between G5 and G5L samples might be due to several reasons. In fact, although has been used the same ingredients at the same concentrations as in the brewery, the yeast strain used by the brewery was in a lyophilized form (not grown on YPD for 72 hours) and the second fermentation took place in a closed bottle in the brewery. Furthermore, the time elapsed from bottling to sampling was not known.

Factors affecting NR, NMN and NAD$^+$ production during the fermentation process

Effect of hop on the production of NR, NMN and NAD$^+$ in a lab-scale produced beer

In order to better define the role of hop in the change of NR and NAD$^+$ levels, two different fermentation trials have been set up: a G5 lab-scale produced beer (MB) and a G5 lab-scale control beer (M1) which did not involve addition of the hop.

Figures 11 and 12 shows the amounts of NR, NMN, and NAD$^+$ in MB and M1, respectively.

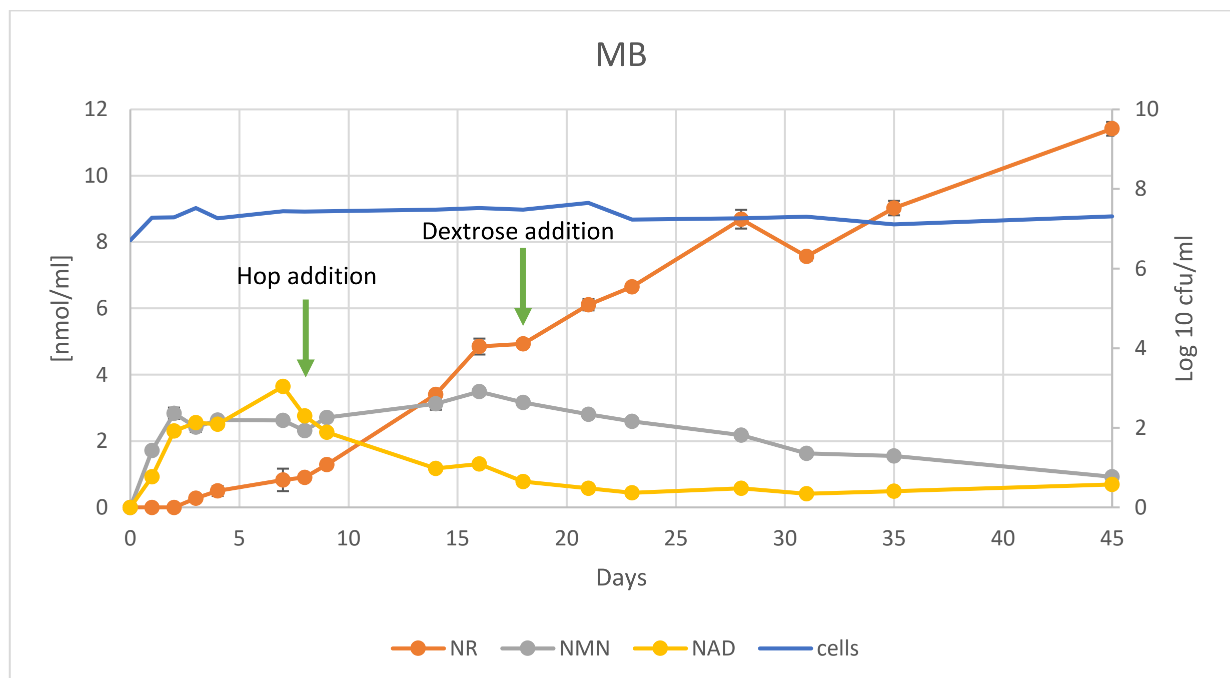


Figure 11. Concentrations of NR, NMN, NAD$^+$ and viable cells in MB during the fermentation process.

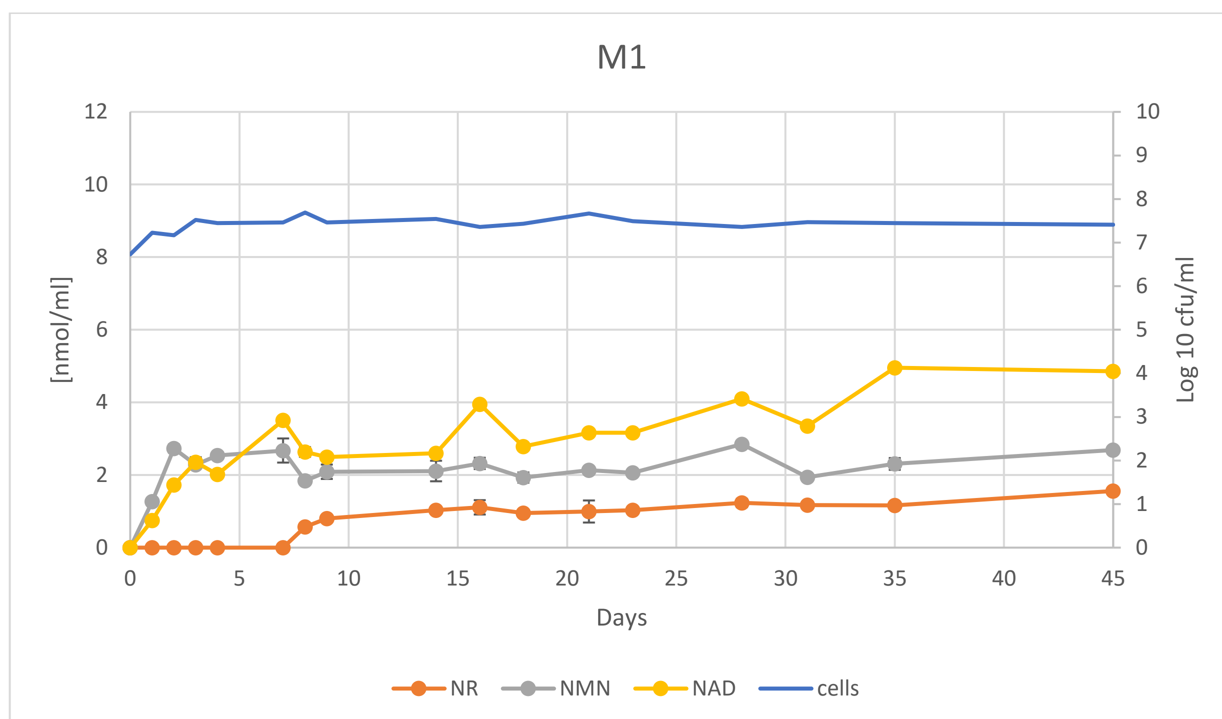


Figure 12. Concentrations of NR, NMN, NAD^+ and viable cells in M1 during the fermentation process.

At the 2nd day of fermentation the concentration of the yeast strain reached the value of 7.5 Log cfu mL^{-1} which remained constant until the end of the process.

Comparing the results of the lab-scale beers shown in Figures 10 and 11 (G5L and MB, respectively), have been found similar trends of NR NMN and NAD^+ even if there are differences regarding their amounts. In fact, their concentrations after the same number of days of fermentation are higher in MB than G5L, especially for NR.

Comparing the amount of the molecules of interest in MB and its control M1, have been found that the amounts of NR are similar in the two samples until the 8th day when the hop was added. On that day, in the MB sample there was a boost of NR, whereas in M1, NR remained stable. This seems to confirm that hop plays a role in the production of NR during the fermentation process. As observed in G5L, the addition of sugar at the 16th did not change the positive trend of NR.

As for NMN, while in MB its amount increased in the first two days, then remained stable and subsequently decreased, in M1 it remained constant throughout the fermentation process.

NAD^+ increased in the early days and had a peak before the addition of hop, and from this step on there was a decrease that led to close to zero. On the other hand, in M1 the amount of NAD^+ increased during the whole fermentation.

Based on these results, hop seems to be responsible for decreasing the amount of NAD^+ and at the same time for boosting NR during the brewing process.

Determination of extracellular activities consuming NAD^+ and producing NR during the fermentation process

It might be hypothesized that the addition of hope might stimulate some extracellular enzymatic activities capable of converting NAD^+ into NR. To this end, aliquots of medium removed at the 23rd and 28th days of fermentation from MB were assayed for the presence of NAD^+-consuming activities by using the HPLC assay as described in paragraph "HPLC assay for the determination of NAD^+-consuming activities". Figures 13, 14 and 15 show the chromatograms of the assay and control mixtures prepared with the different samples after different times of incubation.

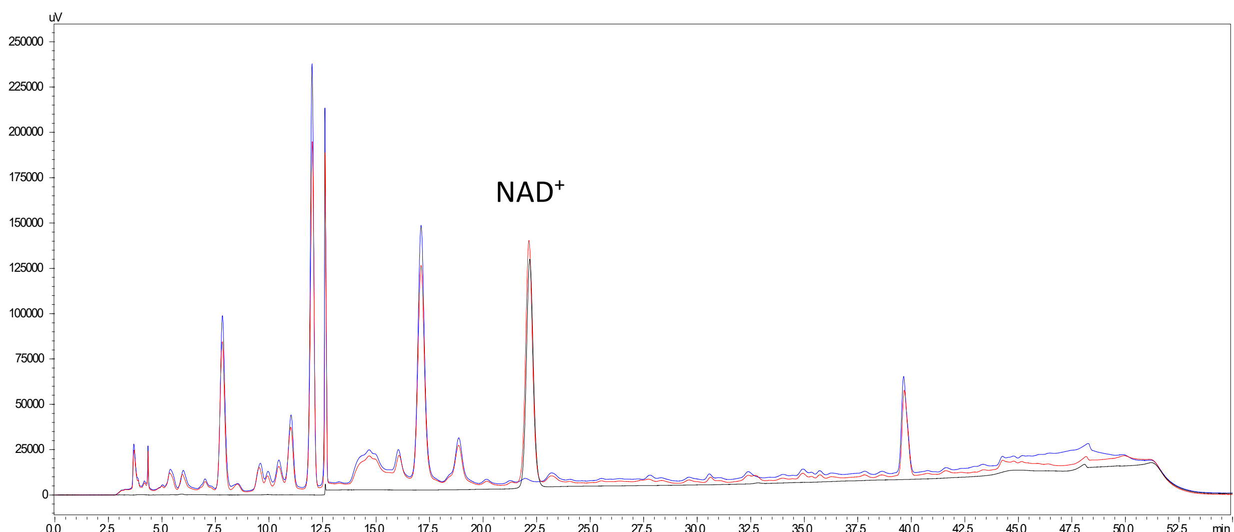


Figure 13. Chromatograms of assay (black) and control (blue, without NAD^+; black, without sample) mixtures of medium at 23rd day of the fermentation process after 24 hours of incubation.

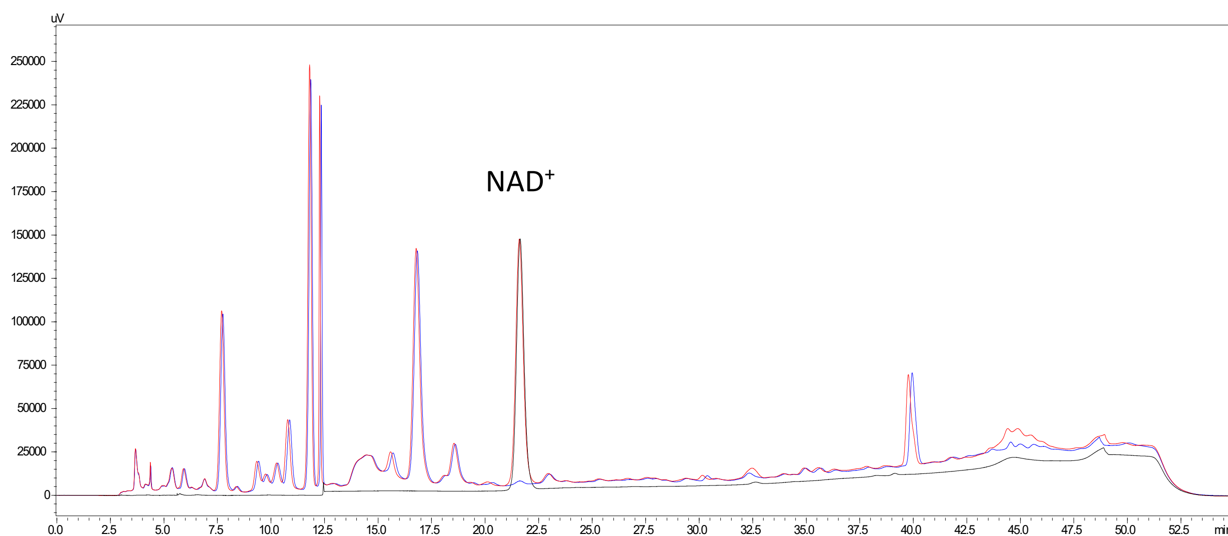


Figure 14. Chromatograms of assay (black) and control (blue, without NAD^+; black, without sample) mixtures of medium at 23rd day of the fermentation process after 48 hours of incubation.

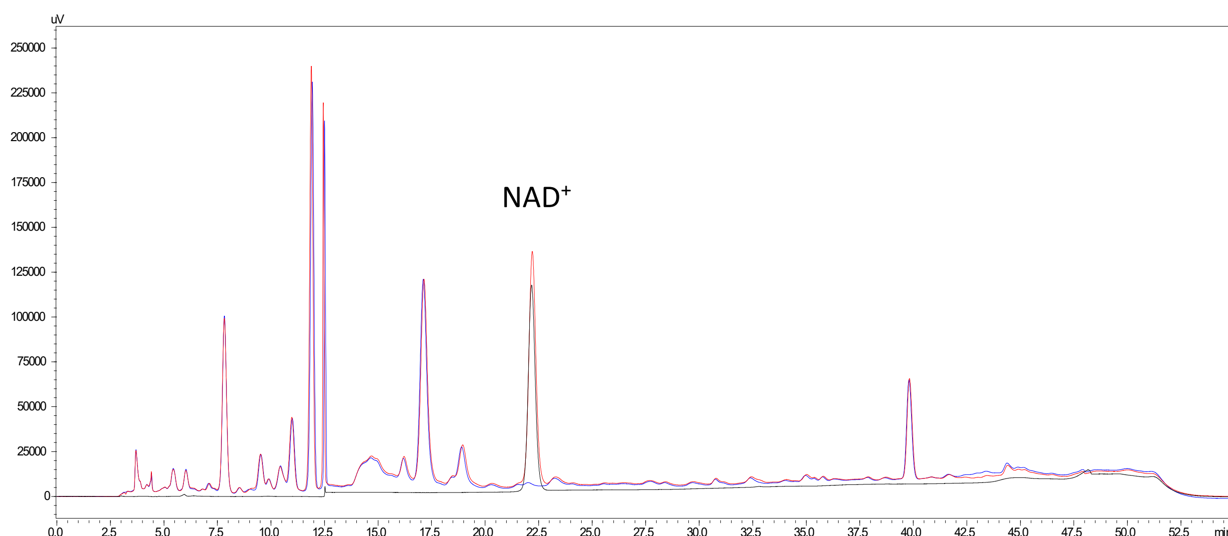


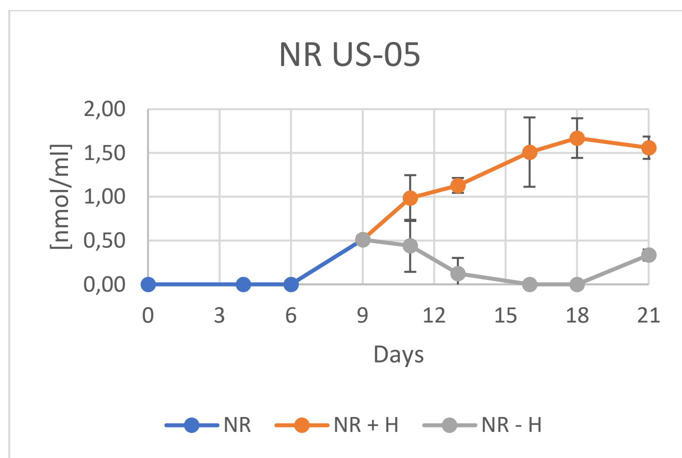
Figure 15. Chromatograms of assay (red) and control (blue, without NAD^+; black, without sample) mixtures of medium at 28th day of the fermentation process after 3 hours of incubation.

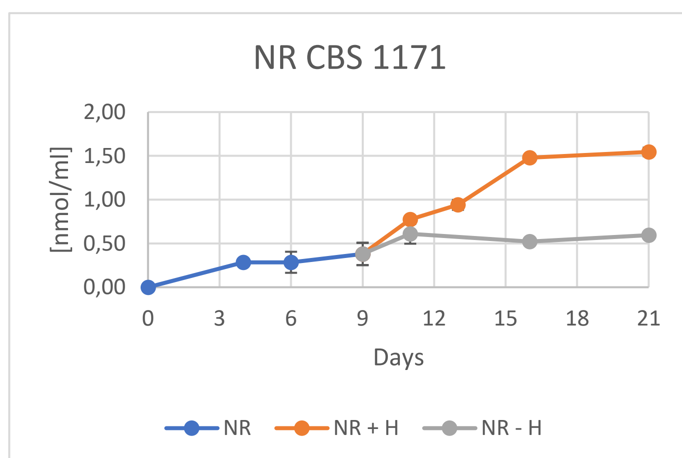
The chromatograms show no differences in the area of the NAD^+ peak in the assay and control mixtures, indicating that in the media there are no enzymes capable to consume NAD^+ during the fermentation.

Effect of wort and *S. cerevisiae* strain on the production of NR, NMN and NAD$^+$ in a lab-scale produced beer

It was investigated whether the presence of the wort was required for the NR bursting effect exerted by the hop. Moreover, it was investigated whether the presence of the yeast was required for the effect of the hop and, in this case, whether the effect would have been strain-specific. Figure 16, 17 and 18 show the amounts of NR, NMN and NAD$^+$ measured in YPD inoculated with *S. cerevisiae* strain US-05 or CBS 1171, from the 1st to the 21st day of fermentation, both with the addition of hop and without it. Moreover, the Figures show the amounts of the three molecules in YPD medium, without the inoculation of *S. cerevisiae* strain (control).

At the 4th day of fermentation in the two fermentations the concentration of the yeast strain reached the value of 7.5 Log cfu mL^{-1} which remained constant until the end of the process (not shown).





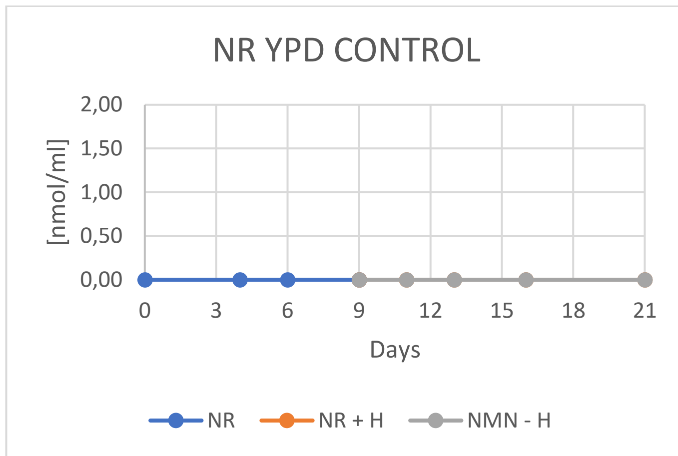
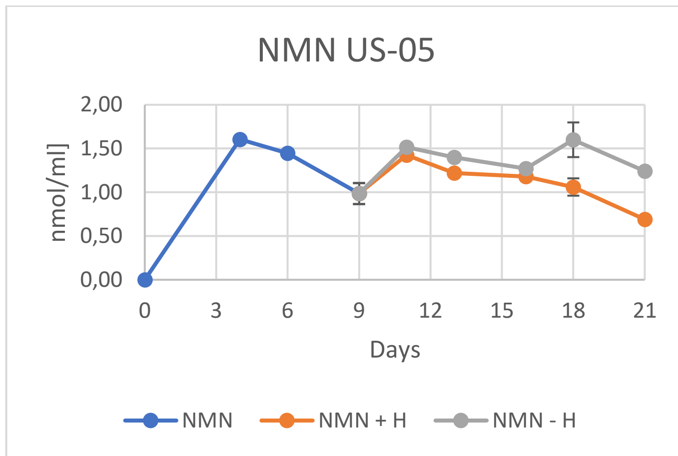
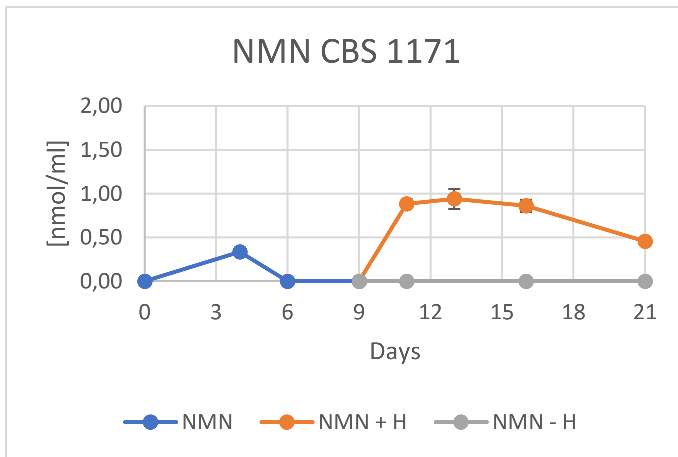


Figure 16. Amount of NR in YPD inoculated with *S. cerevisiae* strain (US-05 or CBS 1171) and in YPD without inoculum from 1st to 21st day of fermentation, before the addition of hop (from the 1st to the 9th day; NR in blue) after the addition of hop (from the 9th day; NR +H, orange) and without the addition of it (from the 9th day; NR -H, grey).

A similar tendency of the vitamin to increase following the addition of hop was observed in both fermentations with the two yeast strains (Figure 16). The difference is related to the fact that CBS 1171 released NR already at the 3rd day, while the strain US-05 only at the 9th day. Moreover, in the absence of hop, NR decreased in the US-05-sustained fermentation, while it continued to slowly increase when CBS 1171 was present. The YPD used as a control shown the absence of NR, indicating that both hop and yeast are essential for boosting NR.

These results also show the discovery of another *S. cerevisiae* strain capable to release NR in YPD medium and that this production seem to be not strain-dependent. Moreover, these results confirm the role of hop in inducing NR production. In addition, they indicate that the presence of wort is not essential for the production of the vitamin.





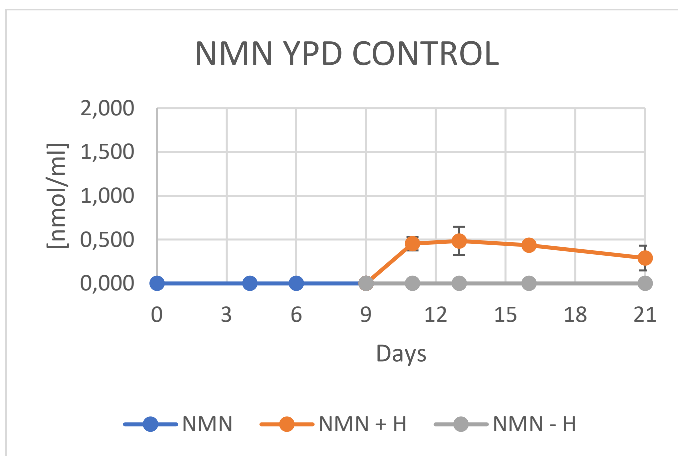
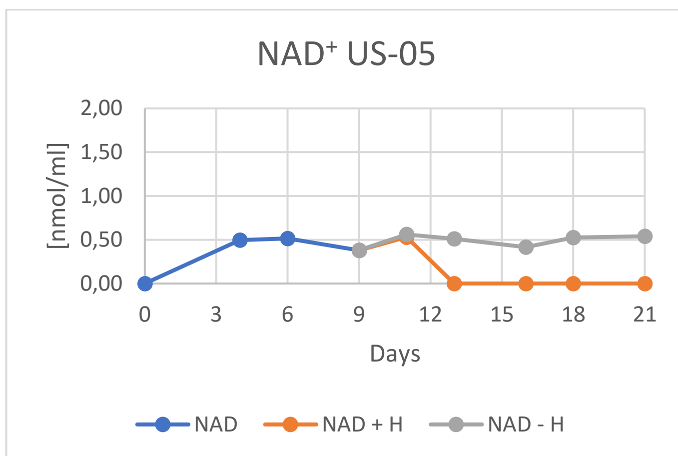


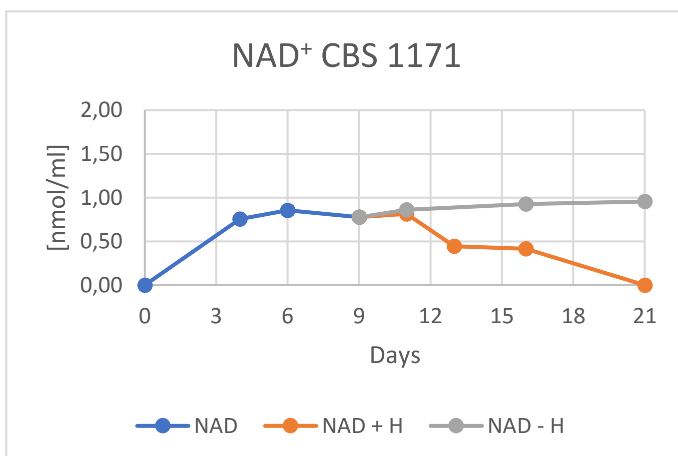
Figure 17. Amount of NMN in YPD inoculated with *S. cerevisiae* strain (US-05 or CBS 1171) and in YPD without inoculum from 1st to 21st day of fermentation, before the addition of hop (from the 1st to the 9th day; NR in blue) after the addition of hop (NR +H, orange) and without the addition of it (from the 9th day; NR -H, grey).

NMN production was different in the two fermentations (Figure 17). The strain US-05 released NMN from the beginning of the process and the addition of hop did not change its concentration up to the

15th day and then it caused a progressive decrease. On the other hand, strain CBS 1171 produced NMN only after the addition of hop, and its amount slowly decreased during fermentation. Furthermore, a low amount of NMN was also detected in the YPD control after the addition of hop, suggesting that hop contains some NMN.

The trend of NAD^+ production was similar in the two fermentations (Figure 18). In fact, in the absence of hop NAD^+ concentration was stable over the time, and the addition of hop caused a decrease of the level, more rapid with the strain US-05. Moreover, the strain CBS 1171 released a higher amount of NAD^+ than US-05. The YPD controls were negative for the presence of NAD^+.





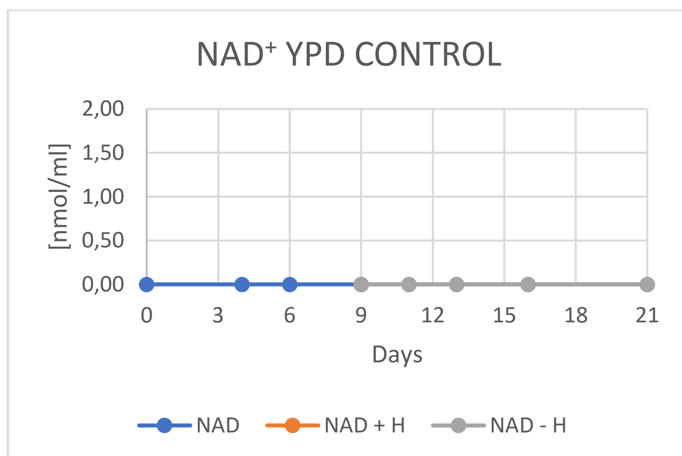


Figure 18. Amount of NAD^+ in YPD inoculated with *S. cerevisiae* strain (US-05 or CBS 1171) and in YPD without inoculum from 1st to 21st day of fermentation, before the addition of hop (from the 1st to the 9th day; NR in blue) after the addition of hop (NR +H, orange) and without the addition of it (from the 9th day; NR -H, grey).

All together these result indicate that both hop and yeast are essential for boosting NR and this effect seems to be strain-independent. These results seem to confirm the role of hop in decreasing the amount of NAD^+ and in increasing NR concentrations, while its addition seems to provide NMN to the medium that can be increased by yeast metabolism. Moreover, this effect seems to be *S. cerevisiae* strain-and wort-independent, as both strains (US-05 and CBS 1171) behave the same in YPD and US-05 has the same effect both in wort and YPD. The interaction among hop and *S. cerevisiae* cells deserves to be further investigated in order to try to develop a low alcohol beer with a high vitamin B_3 content.

Conclusions

The health benefits related to the administration of NR and its precursors (NMN and NAD^+) are well known, therefore it is important to understand the distribution of these molecules in foods and beverages in order to increase their amount. Considering that it is known that *S. cerevisiae* can actively secrete NR, its presence in beer has been hypothesized. To this end, several craft beers were analysed, and the data obtained confirmed the initial hypothesis of the presence of vitamin B_3 in beer. Moreover, the data from the screening of 10 craft beers shown that NAD^+ production is species-dependent, while NR and NMN productions are *S. cerevisiae* strain-dependent. The data obtained from a laboratory-scale beer production shown the potential role of hop in enhancing the NR production within the beer production. In detail, the addition of hop during the beer fermentation process seems to enhance the ability of *S. cerevisiae* to release NR and at the same time to reduce the amount of NAD^+ in the final product. However, further analyses by using an HPLC assay, have demonstrated that these two events are not related to each other. Furthermore, the ability of hop in enhancing the NR secretion by *S. cerevisiae* cultures was confirmed by performing fermentations trials in YPD medium and these further experiments suggested that the ability of releasing NR by *S. cerevisiae* is strain-independent and wort-independent too. Further analyses could be carried out in order to better elucidate how hop interfere with the *S. cerevisiae* metabolism with the final aim to produce a low alcohol beer with a high vitamin B_3 content.

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Chapter 3 - Characterization of kefir grains from Sarajevo (Bosnia and Herzegovina) and their exploitation in traditional vs backslopping methods for kefir production: diversity in microbial dynamics, nutritional and volatilome profiles

Introduction

Kefir

Kefir is a slightly viscous, self-carbonated, acid and low alcoholic fermented milk beverage with a unique volatile profile and nutritional composition (Prado et al., 2015; Gao and Li, 2016). Since a long time ago, kefir has been consumed within the Caucasian and Balkans communities and its daily consumption has been associated with health benefits and longevity (Farnworth, 2005; Kabak and Dobson, 2011; Guzel-Seydim et al., 2011; Gao et al., 2012; Leite et al., 2012, 2013 a, b; Diosma et al., 2014; Pogačić et al., 2013). Nowadays, it is manufactured under a variety of names (kephir, kiaphur, kefer, knapon, kepi, kippi) in several countries such as Argentina, Taiwan, Portugal, Turkey and France, Iran, Poland, Spain, Brasil, China, Norway, Italy (Farnworth, 2005; Magalhães et al., 2011; Grønnevik et al., 2011; Garofalo et al., 2015; Liu et al., 2019).

Traditionally, the kefir production is performed by using kefir grains. These are composed by a natural matrix of exopolysaccharides [EPS (kefiran)] and proteins. Lactic acid bacteria (LAB), yeasts and sometimes acetic acid bacteria (AAB) coexist in this matrix as a symbiotic association (Kabak and Dobson, 2011; Leite et al., 2013 a; Pogačić et al., 2013; Garofalo et al., 2015; Prado et al., 2015). Kefir can also host several health-associated microorganisms (Diosma et al., 2013). At the artisanal/homemade level, kefir grains represent the starter culture for kefir production. Beside microbial cultures kefir contains a mixture of different metabolic compounds as: lactic and acetic acids, carbon dioxide, ethanol, acetaldehyde, acetoin, other volatile compounds, EPS, minerals,

essential amino acids, vitamins (B_1, B_2, B_5, B_{12}, K and C), folic acid, bacteriocins, bioactive peptides, and some nutraceutical components (Farnworth, 2005; Kabak and Dobson, 2011; Guzel-Seydim et al., 2011; Leite et al., 2013 a; Ahmed et al., 2013; Arslan, 2015; Prado et al., 2015). Kefir grains appear as irregular lobed-shaped gelatinous and slimy structures, with variable dimension (from 1 to 4 cm in length) and colour (from white to light yellow) (Kabak and Dobson, 2011; Leite et al., 2013 a; Pogačić et al., 2013; Garofalo et al., 2015; Prado et al., 2015). Traditionally, kefir grains are added to milk in variable ratio (generally from 1 to 20% w/v) and are left to ferment for 18-24 hours at 20-25°C (Leite et al., 2013 a). This method of production utilizes a complex microbiota which plays a fundamental role in the health benefits related to kefir (Leite et al., 2013 a; Bourrie et al., 2016). This microbiota may depend by several factors such as: ratio of kefir grains in milk, time and temperature of fermentation and type of milk used for the production (Altay et al., 2013; Bourrie et al., 2016). In fact, kefir is manufactured by using cow's milk, even if it has also been produced from sheep, goat, buffalo, camel milk and from rice, coconut and soy drinks. In brief, various factors contribute to produce different kefir grains with a different complex microbiota, responsible of microbiological, physico-chemical, nutritional and sensorial properties of final kefir products (Bengoa et al., 2018). During fermentations, kefir the grains are able to increase their biomass and release viable cells into milk (Marshall and Cole, 1985; Prado et al., 2015). Moreover, they can break up into new smaller grains. When the fermentation has terminated, the grains and the kefir are separated, and therefore kefir grains can be used for a next fermentation (Leite et al., 2013 a).

At commercial level, kefir can be produced by using commercial starter strains to obtain a more standardized product with desirable characteristics. Starter cultures containing freeze-dried lactic acid bacteria and yeasts from kefir grains are commercially available. However, healthy benefits have been proved to be linked only to kefir produced with the traditional method (Leite et al., 2013 a; Bourrie et al., 2016).

More recently, kefir is also manufactured by using a different commercial method called "Russian method", in which kefir can be produced at larger scale through backslopping. This is a process of fermentation in series that starts by using kefir produced with kefir grains as natural starter cultures for the next fermentation step (Leite et al., 2013 a; Prado et al., 2015). The backslopping technique allows the scale-up of the production of a kefir drink, using the traditional kefir as starter culture. Moreover, the backslopped kefir contains a microbiota more similar to the traditional kefir than the kefir produced with commercial starter culture. Therefore, backslopped kefir maintains the physico-chemical, nutritional and microbiological characteristics of traditional kefir and its related health benefits.

Health benefits related to kefir

Fermented dairy products have long been associated with the ability to provide health benefits to those who consume them regularly. Metchnikoff in 1908 suggested that fermented milk had a beneficial value and reported it in his theory of longevity. Although not as widely popular as other fermented dairy products, such as yogurt and cheese, kefir has been consumed and associated with health benefits since hundreds of years. Considering that there is a growing trend to consume nutritional food products with some health benefits worldwide, the popularity of kefir has increased in recent years.

Antimutagenic role of microflora in kefir is an established fact. Different strains of bacteria *(Lactobacillus, Streptococcus, Leuconostoc* and *Lactococcus lactis* subp. *cremoris*) isolated from kefir have the capacity to bind mutagens (Hosono et al., 1990; Miyamoto et al., 1991). Kefiran either isolated from kefir grain or produced by *Lactobacillus kefiranofaciens* (a strain isolated form kefir), also have antitumor activity (Wang et al., 2008 a). Different studies have demonstrated that the intake of fermented milk products may lessen the risk of breast cancer in woman (Reddy et al., 1983; Veer

et al., 1989; De Moreno et al., 2007). This depends by the activity of bioactive components, including proteins and small peptides, developed during milk fermentation. These compounds are able to prevent cancer cells' growth (Chen et al., 2007; De Moreno et al., 2007). Moreover, milk proteins and especially sulfur containing amino acid, play a role in kefir, providing anticarcinogenic activity in kefir (Guzel-Seydim et al., 2003). Several other studies have demonstrated that the antitumoral activity of kefir is related to the presence of some polysaccharides in kefir extracts (Ahmed et al., 2013). Water-soluble polysaccharides appeared more effective for tumors suppression than water-insoluble polysaccharides (Furukawa et al., 2000). Moreover, water-soluble polysaccharides effectiveness against tumors also improved at higher dosage level (Murofushi et al., 1983). These compounds seem to act in different sites in different ways. Overall, various studies show the great potential of these bioactive compounds as anticarcinogenic molecules.

Peptides produced during milk fermentation or during digestion have demonstrated to stimulate the immune system in animal models (LeBlanc et al., 202; Matar et al., 2003). Kefir have an immunomodulatory and prodigestive effect (Jianzhong et al., 2009). A study has demonstrated that kefir milk products and fermented dairy products have a capacity to induce powerful mucosal response for boosting immunity thus maintaining the homeostasis in intestine (Vinderola et al., 2006). Kefir microbiota and exopolysaccharides of kefir grains have demonstrated to stimulate the immune system in animal models (LeBlanc et al., 2002; Matar et al., 2003; Murofushi et al., 1983; Murofushi et al., 1986; Thoreux and Schmucker, 2001).

Kefir microbiota is able to produce and release a wide range of antimicrobial compounds such as hydrogen peroxide, peptides (bacteriocins), ethanol, carbon dioxide, diacetyl and organic acids (lactic and acetic acids) that can be exploited not only reducing foodborne pathogens and spoilage bacteria during food production and storage, but also in the treatment and prevention of gastrointestinal disorders and vaginal infections (Zamfir et al., 1999; Bonadè et al., 2001; Messens and De Vuyst, 2002; Czamanski et al., 2004; Jamuna and Jeevaratnam, 2004; Powell et al., 2007; Liu et al., 2008;

Zhou et al., 2008; Simova et al., 2009). These compounds have demonstrated antimicrobial activity against *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Salmonella enteridis*, *Shigella flexneri* and *Yersinia enterocolitica* (Santos et al., 2003). This bactericidal effect is related both bacteria and yeast and may be attributed to activity of undissociated acetic acid, lactic acid and H2O2. (Yuksekdag et al., 2004 a; Yuksekdag et al., 2004 b).

A part of the world population is unable to digest lactose, because of insufficient intestinal β-galactosidase activity. However, kefir grains have shown to possess β-galactosidase activity which remains active when consumed, and commercial kefir produced using starter cultures has demonstrated to be able to reduce the lactose content of milk (De Vrese et al., 1992; Hertzler and Clancy, 2003). These results show that kefir is a milk fermented beverage suitable for lactose-intolerant person.

Kefir grains and yeasts isolated from them are able to provide a hypocholesterolemic effect (Vujicic et al., 1992; Tamai et al., 1996). High amount of lactic acid bacteria in kefir ensures the binding of cholesterol through their metabolic products (Hosono and Tanako, 1995). It has been noticed that when milk was inoculated with kefir cultures at 24.8°C and incubated for 24 h, it resulted in an assimilation of cholesterol by 28-65% (Vujicic et al., 1992). These results show that kefir and its components have greater potential to be used as hypocholesterolemic substance (Maeda et al., 2004; Liu et al., 2006).

Besided, kefir and its exopolysaccharides have shown anti-inflammatory properties by inhibiting the formation of granuloma tissue (Rodrigues et al., 2005) and antiallergic properties that could be used to treat allergic bronchial asthma (Lee et al., 2007).

Kefir contains high folate content, related to yeasts (Zubillaga et al., 2001; Patring et., 2006). In fact, yeast strains isolated from kefir have showed a high folate producing capacity (Moslehi-Jenabian et al., 2010).

Furthermore, the antioxidative properties of kefir (Chen et al., 2006) and its capacity to reduce blood glucose and blood pressure (Maeda et al., 2004) and to protect against apoptosis have been proved (Nagira et al., 1999; Matsuu et al., 2003).

Object of the work

Surprisingly, although kefir has been originated from Balkans, to the author's knowledge, few scientific studies have been published on microbiota characterization of kefir grains from this area (Bulgaria; Simova et al., 2002), (Turkey; Guzel-Seydim et al., 2005; Kesmen and Kacmaz, 2011; Kök Taş et al., 2012) and none from Bosnia and Herzegovina. Furthermore, relatively few studies have been published on the comparison of the microbiota between kefir grains and the related kefir drinks obtained through traditional method (Guzel-Seydim et al., 2005; Gao and Zhang, 2019; Korsak et al., 2015; Marsh et al., 2013; Dobson et al., 2011; Kesmen and Kacmaz, 2011) and none on the full characterization of the product and microbial dynamics occurring during the production of kefir through backslopping method.

Based on these observations - and considering the beneficial effects of the kefir related with its microbiota – a study has been performed about kefir grains collected from Bosnia and Herzegovina and the related kefir drinks (traditional and backslopped). The study aims to obtain elucidations: i) on the bacterial and fungal community composition of five kefir grains collected in Sarajevo (Bosnia and Herzegovina); ii) on the microbial dynamics occurring during the production of kefir through traditional and backslopping methods by viable counts on selective culture media and amplicon based sequencing, iii) on the characterization of the related kefir drinks in terms of chemical, nutritional and colorimetric features and volatilome profile.

This study has been performed in collaboration with the following partners: i) DISAFA - Microbiology and food technology sector, University of Turin; ii) Istituto di Scienze dell'Alimentazione, CNR; iii) Faculty of Agriculture and Food Sciences, University of Sarajevo; iv) Dipartimento Agricoltura, Ambiente e Alimenti, Università degli Studi del Molise.

This research has been submitted to the Food Microbiology journal.

Material and methods

Origin, maintenance of milk kefir grains and kefir production

Five milk kefir grains (KGA, KGB, KGC, KGD, KGE) were obtained from private households located in Sarajevo (Bosnia and Herzegovina). Kefir grains were activated in laboratory as following described. Kefir grains were inoculated (10% w/v) in sterile whole bovine (U.H.T.) milk and incubated at 22 °C for 24 h. After this time, the grains were filtered through a sterilized plastic sieve, they were gentle washed with sterile distilled water and subsequently, the grains were inoculated again in milk for a daily fermentation at 22 °C. This procedure was repeated for 1 month in order to keep the grains active and to increase the grains biomass.

Five kefir drinks were produced by traditional method (by using each kefir grain) and by backslopping method (by using the fermented milk obtained from each kefir grain fermentation) as follows. Twenty grams of each grain were inoculated (10% w/v) in 200 mL sterile whole bovine (U.H.T.) milk (12.14% dry matter; 3.74% fat; 3.35% protein; 4.65% lactose; and 0.74% ash) and incubated at 22 °C for 24 h.

After incubation, the grains were separated by filtration from the fermented milks. The filtrate that correspond to the traditional kefir (hereafter referred as TK) was also used as natural starter culture to produce kefir by backslopping (hereafter referred as BK). In detail, aliquots of 50 ml of each TK was used for inoculating 1 L of U.H.T sterile whole bovine milk (5% v/v). The fermentation was statically performed at 22 °C for 24 h (Satir et al., 2016; Puerari et al., 2012). The preparation procedure for TK and BK is reported in Figure 1. Kefir production was carried out in duplicates (Puerari et al., 2012), and considered as Blocks in the data analysis.

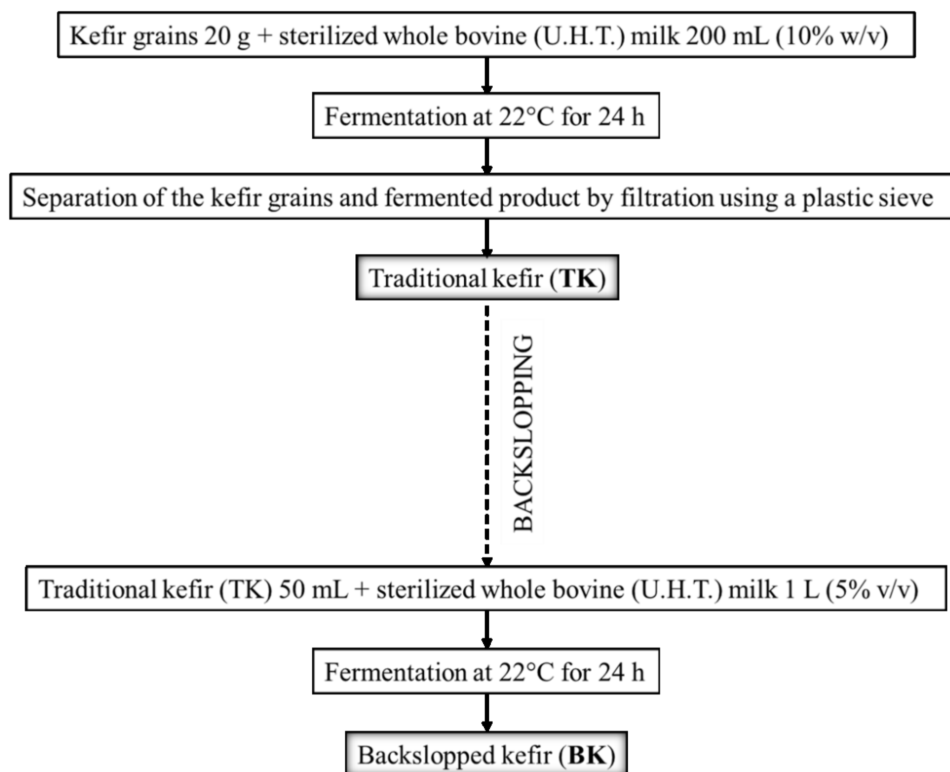


Figure 1. Flow diagram of manufacture of traditional and backslopped kefir

Enumeration of culturable bacteria and yeasts in milk kefir grains and drinks

Ten grams of each kefir grain (KGA-KGE) were homogenised in 90 mL of cold sterile 0.1% peptone solution using a Stomacher apparatus (400 Circulator, International PBI) for 15 min at maximum speed (Kołakowski and Ozimkiewicz, 2012; Garofalo et al., 2015).

Ten milliliters of each kefir drink: traditional kefir (TKA-TKE) and backslopped kefir (BKA-BKE) were diluted in 90 mL of cold sterile 0.1% peptone solution.

All of the samples were serially diluted. Serial decimal dilutions were prepared in cold sterile 0.1% peptone solution and 0.1 mL of each serial dilution was inoculated by surface spreading onto specific solid media in duplicate. The following microorganisms were counted: (i) lactobacilli on MRS agar (Difco, Sparks, MD, USA) at 37 °C under anaerobic conditions (Aquilanti et al., 2012); (ii) AAB on Gluconobacter Medium (GM) agar (mannitol 2.5%, yeast extract 0.5%, peptone 0.3%) (Gulitz et al.,

2011); all the above media were supplemented with 400 mg/L of cycloheximide to inhibit yeast growth (Camu et al., 2008); (iii) yeasts on Rose Bengal Chloramphenicol Agar (RBCA) (Difco) at 25 °C aerobically. Microbial enumerations of bacteria and yeasts were carried out after 3 and 10 days, respectively.

The results of the viable counts were expressed as means of the log of colony forming units (cfu) per gram of sample.

DNA extraction from milk kefir grains and drinks

The microbial DNA was extracted directly from the milk kefir grains (KGA-KGE) and kefir drinks (TKA-TKE and BKA-BKE) using PowerFood™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA). In detail, 1 mL of each kefir grain homogenate (dilution 10^{-1}) used for microbial plating was centrifuged at 13.000 g for 3 min to produce a pellet, while 1.5 mL of each kefir drinks was centrifuged at 13.000 g for 3 min. Each pellet (from kefir grains and kefir drinks) was processed according to the kit manufacturer's instructions. The DNA quantity and purity were assessed by optical readings at 260, 280 and 234 nm, respectively, using a UV-Vis Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Analysis of bacterial and yeast diversity by rRNA gene Illumina sequencing and bioinformatics and data analysis

Amplicon target sequencing

DNAs directly extracted from kefir grains (KGA-KGE) and kefir drinks (TKA-TKE and BKA-BKE) were quantified using a QUBIT dsDNA Assay kit (Life Technologies, Milan, Italy) standardized (20

ng/ µL) and used to amplifying the V3-V4 region of the 16S rRNA (Klindworth et al., 2013) as well as the D1 domain of 26S rRNA gene (Mota-Gutierretz et al., 2018).

PCR was performed for the two target regions using a PCR mixture as follow: 12.5 µL of the 2× Kapa HiFi HotStart ReadyMix Taq (Roche, Milan, Italy), 5 µL of each primer (1 µM), 2.5 µL of DNA as a template. PCR were subject to the following amplification conditions: thirty cycles of 30 s of denaturation (95 °C), 30 s of primer annealing (55 °C), and 30 s of primer elongation (72 °C), followed by a final elongation step (72 °C) of 10 min.

PCR amplicons were purified and sequenced according to the Illumina metagenomic pipeline instructions. The sequencing was performed with a MiSeq Illumina instrument (Illumina) with V3 chemistry and generated 250 bp paired-end reads according to the manufacturer's instructions.

Bioinformatics analysis

After sequencing, raw reads were merged with FLASH software (Magoc and Salzberg, 2011) and the resulting reads were analyzed through QIIME 1.9.0 software (Caporaso et al., 2010) and the pipeline recently described (Ferrocino et al., 2017). For 16S data, Centroids sequences of each OTUs cluster were mapped against the Greengenes 16S rRNA gene database version 2013 for taxonomic assignment, while for 26S data the in-house database from Mota-Gutierrez et al. (2018) was used.

In order to avoid biases due to the different sequencing depth, each dataset was rarefied at lowest number of reads: 26S data were rarefied at 37376 sequences while 7969 sequence were chosen for 16S data. The OTUs tables generated through QIIME showed the higher taxonomy resolution that was reached. Sequences were double-checked using the BlastN search tool (http://www.ncbi.nlm.nih.gov/blast/) to confirm the taxonomy assignment.

Chemical measurement of kefir drinks

The pH of kefir drinks (TKA-TKE and BKA-BKE) was measured at room temperature using a model 300 pH meter equipped with an HI2031 solid electrode (HI2031, Hanna Instruments, Padua, Italy).

For the Total Titratable Acidity (TTA) determination, 10 mL of kefir drinks were mixed with 90 mL of distilled water, stirred by magnetic stirrer and then titrated with 0.1 N NaOH. The results were expressed as the mean amount of the NaOH (mL) used to reach the pH 8.3. All the assays were carried out in triplicate.

Proximate composition of kefir drinks

Kefir drinks (TKA-TKE and BKA-BKE) and milk were analyzed for moisture, dry matter, fat, protein, anhydrous lactose and ash content. The analyses were carried out in the same accredited laboratory (ACCREDIA, accreditation No. 0217). Fat %, protein %, anhydrous lactose %, expressed as w/w, were quantified via Fourier Transform Infrared (FTIR) spectroscopy using a CombiFoss FT+ composed by Milkoscan FT Plus – 300 and Fossomatic FC (Foss Electric, Hillerød, Denmark); dry matter and ash according to AOAC methodologies (950.46; 920.153). All the chemical analyses were carried out in duplicate and the results were expressed as % (w/w).

D-/L-lactic acid determination

The concentration of D-/L-lactic acid of the kefir drinks (TKA-TKE and BKA-BKE) was quantified by using a Megazyme Assay Kit (K-DLATE 11/17) (MEGAZYME International, Wicklow, Ireland, 2017) following the manufacturer’s instructions. All samples were examined in triplicates.

Colour properties of kefir drinks

Colorimetric profile of kefir drinks (TKA-TKE and BKA-BKE) was measured by a Chroma Meter CR-200 (Minolta, Japan) to determine L (lightness) and chromaticity coordinates (a*, b* expressing redness and yellowness, respectively) according to CIELab. Color intensity was determined by calculating Chroma index according to the following equation: $\sqrt{(a^{*2} + b^{*2})}$. The set-up of Chroma Meter was performed on a white reference standard plate with a D65 illuminant, characterized by a visible spectrum similar to the natural light. The colorimetric readings were performed in triplicate for each sample.

Volatile organic compounds (VOCs) of kefir drinks

Characterization of volatile organic compounds (VOCs) of kefir drinks

The volatile fraction of kefir drinks (TKA-TKE and BKA-BKE) was analyzed by headspace sampling, using Solid Phase Micro-Extraction coupled with Gas Chromatography/Mass Spectrometry (SPME-GC/MS). In detail, for each SPME analysis, 5 g of samples were placed into 20 mL headspace vial, and immediately sealed with a Teflon-lined septum and screwcap. The vial was placed in a thermostatic block on a stirrer at 55 °C for 5 min. After equilibration, the headspace of the samples was sampled using an SPME fiber coated with DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane, thickness 50/30 mm) at 55 °C for 50 min. HS-SPME was automatically carried out with a multipurpose sampler (Gerstel MPS2) (Dertli and Çon, 2017).

Gas chromatography/mass spectrometry (GC/MS) analysis

Volatile analysis was performed using an Agilent Technologies 7890A GC coupled to an Agilent Technologies 5975 mass spectrometer (Santa Clara, California) equipped with a 30 m x 0.25 mm ID, film thickness 0.25 μm capillary column (HP-INNOWAX, Agilent Technologies). Gas carrier was helium (flow 1.5 mL/min) and SPME injections were splitless (straight glass line, 0.75 mm ID) at 240 °C for 20 min during which time thermal desorption of analytes from the fiber occurred. The oven parameters were as follows: initial temperature was 40°C held for 3min, followed by an increase to 240 °C at a rate of 5 °C/min, then held for 10 min. Injector temperature was 240 °C. Mass spectrometer operated in scan mode over mass range from 33 to 300 amu (2 s/scan) at an ionization potential of 70 eV. Identification of volatile compounds was achieved by comparing mass spectra with the Wiley library (Wiley7, NIST 05). The amount of the individual compounds was expressed as a peak area percentage obtained by automatic integration of the peak area of the compound/∑peak areas of all compounds identified in the chromatograms. All the analyses were performed in duplicate. The operating conditions utilized were according to Di Renzo et al. (2018).

Statistical analyses

The analysis of variance (ANOVA) of physico-chemical and colorimetric parameters included kefir drink (KD) and kefir drink culture type (KD-CT) as main effects, and their interaction. Kefir drink had two levels: traditional kefir (TK) and backslopped kefir (BK); five different kefir drink culture type (KD-CT) were used and coded as A, B, C, D, E.

Data of physico-chemical and colorimetric parameters were analyzed according to a split-block design, where the two replications of the experiment were considered as Blocks. The significance of the error variances of the main effects (KD; KD-CT) were tested and, in the lack of significance, a

pooled error variance was generated and used to test main effects and interaction variances. Mean comparison between the two level of kefir drink was performed by the Least Significant Difference (LSD) test, whereas multiple comparisons among means were carried out using the Honest Significant Difference (HSD) test for kefir drink culture type and the KD x KD-CT interaction. The same ANOVA model was applied to microbiological plate count variables (LAB, AAB, yeasts), but kefir drink factor had three levels (kefir grains-KG, traditional kefir-TK and backslopped kefir-BK). Multiple comparisons were performed by the Honest Significant Difference (HSD) test using JMP software (version 11.0).

Statistical analyses of volatile compounds were performed using SYSTAT 13.0 for Windows (Systat Software Inc., Richmond, CA, USA). Data were expressed as mean ± standard error of mean. Statistical significance was evaluated by one-way analysis of variance (ANOVA) with Tukey's HSD test. The p-value <0.05 was considered statistically significant. Principal component analysis (PCA) based on the contents of the volatile constituents was performed.

For the sequencing data sample coverage and diversity index (alpha diversity) were calculated using the *diversity* function of the vegan package (Dixon, 2007). For 16S data Unweight UniFrac distance matrix were used to find differences between samples by Anosim and Adonis statistical test through the function *vegan* in R environment. Non-parametric Pairwise Wilcoxon test were used as appropriate to determine significant differences in alpha diversity index, OTU abundance or VOCs composition among the three types of samples. Principal component analysis (PCA) were performed and plotted using the function *dudi.pca* through the *made4* package of R. Pairwise Spearman's correlations between taxa and volatile organic compounds were assessed by the R package *psych*. P values were adjusted for multiple testing using the Benjamini-Hochberg procedure, which assesses the false discovery rate (FDR).

Results and Discussion

Characteristics of kefir grains from Sarajevo

Macroscopic observation of the milk kefir grains collected from Sarajevo showed that all of the grains were lobed and irregular in shape and differed for size and colour. In particular, grain KGA and KGB were the biggest since they span about 4 cm; grain KGC was the smallest (about 1 cm in length) while KGD and KGE were about 2 cm in length. Grain KGC also appeared whiter than the others that showed a light yellow colour. Grain KGE was characterized by a cavity at the center of the grain.

Proximate composition, chemical features, color profile and microbial counts of traditional (TK) and backslopped (BK) kefir

Concerning the statistical analysis of the data obtained from the physico-chemical and colorimetric profile (Table 1), the Blocks x KD and the Blocks x KD-CT variances were always not significant for almost all the variables. Therefore, a single pooled error variance was applied to test main effects and interaction variances for physico-chemical and colorimetric profile parameters.

ANOVA results (Table 1) showed that kefir drink variance was significant for all parameters except for fat and lightness (L) and the KD-CT variance highlighted significant differences for pH, TTA, and D-lactic acid. Moreover, significant KD x KD-CT interaction was detected only for pH and L-lactic acid.

Source of variation	DF	pH	TTA (g/L)	H_2O (%)	D.M. (%)	Fat (%)	Protein (%)	Lactose (%)	Ash (%)	D-lactic Ac (g/L)	L-lactic Ac (g/L)	L	a*	b*	Chroma
Blocks	1	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
KD	1	***	***	**	**	n.s.	**	**	***	***	***	n.s.	*	**	**
KD-CT	4	***	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.
KD x KD-CT	4	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.

Table 1. ANOVA results for chemical-physical and colorimetric parameters of kefir products

DF = degree of freedom, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, n.s. = not significant

KD = kefir drink (traditional and backslopped kefir), KD-CT = kefir drink culture type

	H_2O (%)	D.M. (%)	Fat (%)	Protein (%)	Lactose (%)	Ash (%)	pH	TTA (g/L)	D-lactic Ac (g/L)	L-lactic Ac (g/L)	L	a*	b*	Chroma
TK	89.11^{A}	10.88^{B}	3.40^{A}	3.01^{B}	2.39^{B}	0.66^{B}	3.74^{B}	11.42^{A}	5.79^{A}	2.54^{A}	86.08^{A}	-7.90^{A}	9.10^{B}	12.07^{B}
BK	88.15^{B}	11.85^{A}	3.79^{A}	3.35^{A}	2.92^{A}	0.70^{A}	4.30^{A}	6.82^{B}	1.48^{B}	5.20^{A}	85.49^{A}	-8.48^{B}	11.26^{A}	14.11^{A}
KD-CT A	88.40^{A}	11.60^{A}	3.85^{A}	3.33^{A}	2.45^{A}	0.70^{A}	4.03^{B}	8.65^{AB}	2.90^{B}	4.42^{A}	85.90^{A}	-8.17^{A}	10.46^{A}	13.29^{A}
KD-CT B	88.24^{A}	11.76^{A}	3.73^{A}	3.30^{A}	2.87^{A}	0.67^{A}	4.26^{A}	8.13^{B}	3.22^{B}	3.89^{A}	84.88^{A}	-8.46^{A}	10.48^{A}	13.47^{A}
KD-CT C	88.64^{A}	11.37^{A}	3.59^{A}	3.08^{A}	2.41^{A}	0.69^{A}	3.89^{C}	10.01^{A}	4.86^{A}	3.42^{A}	86.12^{A}	-8.22^{A}	10.37^{A}	13.24^{A}
KD-CT D	89.28^{A}	10.72^{A}	3.28^{A}	2.92^{A}	2.74^{A}	0.69^{A}	3.99^{BC}	8.93^{AB}	3.91^{AB}	3.14^{A}	86.27^{A}	-8.18^{A}	9.63^{A}	12.67^{A}
KD-CT E	88.62^{A}	11.38^{A}	3.54^{A}	3.28^{A}	2.79^{A}	0.68^{A}	3.95^{BC}	9.88^{A}	3.28^{B}	4.49^{A}	85.75^{A}	-7.94^{A}	9.97^{A}	12.77^{A}

Table 2. Chemical-**p**hysical and colorimetric parameters mean comparisons of kefir products (LSD test) and five culture types (HSD test)

Means within the same column with different letters are different at $P \leq 0.05$

TK = traditional kefir, BK = backslopped kefir, KD-CT = kefir drink culture type

A significant increase in dry matter content for BK (11.85%) compared to the TK products (10.88%) was recorded (Table 2). Same trend was observed for protein, lactose, ash content showing significantly higher values for BKs (3.35%, 2.92% and 0.70% respectively) than TKs (3.01%, 2.39% and 0.66%, respectively). Fat content increased in BKs (3.79%) compared to TKs (3.40%), even though this difference was not significant. Overall, the proximate composition of kefir drinks was very similar, for dry matter, protein, ash and lactose contents, to the results referred by Sekkal-Taleb (2016) and reported by Irigoyen et al. (2005) using traditional fermentation method and by Kim et al. (2018) using both traditional and backslopping methods. No significant differences for protein, fat and ash content among TK and BK were detected by Kim et al. (2018), although some other differences in nutritional composition for TK and BK were found. Interestingly, our results for protein content of TKs and BKs (3.01% and 3.35%, respectively) were lower than the ones reported by Satir and Seydim (2016) that, applying the same fermentation methods, analyzed kefirs manufactured using milks from other mammalian species. As expected, the lactose content of the two kinds of kefir drinks (TK and BK) was lower than in the raw milk (4.65%), and this reduction rate was higher respect to values obtained by Irigoyen et al. (2005) and Garcia Fontan et al. (2006).

The highest pH values in BK samples respect to TK samples were related to a minor TTA values in BK. An increase of pH from TK to BK was also reported by Kim et al. (2018). The pH value of BK (4.30; Table 2) was the same reported by Kim et al. (2018) for TK and this value is indicated by Gao and Li (2016) as one of the best chemical characteristics of kefir.

D- and L-lactic acids content showed opposite trends in TK and BK: TK presented a significantly higher D-lactic acid content (5.79 g/L) than BK (1.48 g/L), whereas a not significant difference of L-lactic acid was observed between BK (5.20 g/L) and TK (2.54).

The comparison among the means of the five KD-CT showed that culture type B had a significantly higher pH level (4.26) then all the other cultures. Significant differences were also detected among culture types for TTA; culture type C had a significantly higher D-lactic acid content then all other

cultures but D; whereas L-lactic content did not differ among culture types (Table 2). For the remaining variables (compositional and colorimetric) the five culture types resulted very similar since no differences were detected (Table 2).

pH and L-lactic acid content showed a significant interaction between KD and KD-CT. For the pH parameter, multiple comparisons showed that the five culture types had significantly higher pH values in BK than in TK; however, culture type B revealed a much higher increase (3.88 vs 4.63) than all other cultures (Table 3).

	pH	
BK-CT B	4.63	A
BK-CT A	4.25	B
BK-CT D	4.24	B
BK-CT E	4.22	B
BK-CT C	4.16	B
TK-CT B	3.88	C
TK-CT A	3.80	C D
TK-CT D	3.74	C D
TK-CT E	3.66	D
TK-CT C	3.62	D

Table 3. Multiple comparisons among KD x KD-CT interaction means (HSD test) for pH

BK = backslopped kefir, TK = traditional kefir, KD-CT = kefir drink culture type

The KD x KD-CT interaction for in L-lactic acid was mainly due to the significant increase in kefir produced with culture type E from traditional to backslopped kefir; all other cultures showed a not significant increase for this parameter (Table 4).

	L-Lactic acid	
BK-CT E	7.23	A
BK-CT A	5.98	A B
BK-CT B	4.57	A B C
BK-CT C	4.23	A B C
BK-CT D	3.96	A B C
TK-CT B	3.20	B C
TK-CT A	2.86	B C
TK-CT C	2.60	C
TK-CT D	2.31	C
TK-CT E	1.74	C

Table 4. Multiple comparisons among KD x KD-CT interaction means (HSD test) for L-lactic acid

BK = backslopped kefir, TK = traditional kefir, KD-CT = kefir drink culture type

For the colorimetric profile, the two kefir drinks did not differ for the lightness parameter (L); whereas a significantly lower redness index (a*) and a significantly higher b* and Chroma were found for BK. Mean values for L coordinate resulted in lower levels (86.08 and 85.49, respectively for TK and BK) compared to data reported by Gul et al. (2018) for kefirs produced with cow and buffalo milk (91.93 and 92.22) using kefir grains according to traditional methodology, and by Znamirowska et al. (2017) for natural sheep kefir (91.44). The red-green coordinate (a*) showed lower means (-7.90 and -8.48), resulting therefore in lightly greenish color perception, than those reported by Gul et al. (2018) for cow and buffalo kefir (-1.01 and -1.49), and by Znamirowska et al. (2017) for sheep milk kefirs (-4.17). The yellow-blue coordinates (b*) were similar to the ones measured in commercial and traditional cow milk kefir (Gul et al., 2018). Values of saturation index (Chroma), significantly different between kefir products, were higher compared to commercial and traditional cow milk kefir reported by Gul et al. (2018).

Concerning microbial counts, ANOVA results for LAB, AAB and yeast counts are reported in Table 5 showing that all sources of variation were highly significant.

Source of variation	DF	LAB (log_{10} cfu/mL)	AAB (log_{10} cfu/mL)	Yeasts (log_{10} cfu/mL)
Blocks	1	n.s.	n.s.	n.s.
KG-TK-BK	2	***	***	***
KD-CT	4	**	**	***
KD x KD-CT	8	**	***	***

Table 5. ANOVA results for Lactic Acid Bacteria (LAB), Acetic Acid Bacteria (AAB) and yeasts

DF = degree of freedom, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, n.s. = not significant

KG = kefir grains, TK = traditional kefir, BK = backslopped kefir, KD-CT = kefir drink culture type

Multiple comparison for kefir grains and drinks showed a significant increase of LAB and AAB from kefir grains to TK and from this one to BK. Differently from LAB and AAB, the yeast counts progressively and significantly decreased from the grains to the BK (Table 6).

	LAB (log_{10} cfu/mL)	AAB (log_{10} cfu/mL)	Yeasts (log_{10} cfu/mL)
KG	7.99^{C}	7.86^{C}	7.62^{A}
TK	8.75^{B}	8.98^{B}	6.31^{B}
BK	9.30^{A}	9.23^{A}	5.90^{C}

Table 6. Lactic Acid Bacteria (LAB), Acetic Acid Bacteria (AAB) and yeasts mean comparisons (HSD test) of kefir grain (KG) and drinks (TK and BK). Means within the same column with different letters are different at $P \leq 0.05$. KG = kefir grains, TK = traditional kefir, BK = backslopped kefir

This trend is not surprisingly since already Marshall and Cole (1985) reported that when kefir was used as starter culture for subsequent fermentations, the balance of the microbiota in the deriving products was lost since the lactobacilli counts remained high but yeasts counts were progressively reduced. The yeasts number reduction during backslopping process was also recently confirmed by Kim et al. (2018) that also reported yeast counts very similar [7.10 log cfu/g (TK) and 5.22 log cfu/g (BK)] to those found in the present study [7.62 log cfu/g (TK) and 5.90 log cfu/g (BK)] (Table 6). These data can be explained by several studies that reported that yeasts cells are localized on the outer areas of grains, therefore they may easily fall in milk, reducing the rate of growth in milk probably due to the absence of the symbiotic association with LAB and AAB that occurs within grains (Garofalo et al., 2015; Guzel-Seydim et al., 2005; Kesmen and Kacmaz et al., 2011; Pintado et al.,

1996; Gulitz et al., 2011). Kim et al. (2018) underlined some positive effects, from the commercial point of view, related to the decrease of yeasts counts and the increase of pH on the shelf-life of BK. Indeed, on the one hand the reduced numbers of yeasts would result in reduction of the rate of low-temperature fermentation leaded by yeasts, on the other hand the higher pH would result in positive sensory features as flavor and taste during shelf-life of the product (Kim et al., 2018).

For LAB, AAB and yeasts, significant differences were found among the five culture types as showed in Table 7. In particular culture type C had the highest count levels for the three microbial parameters.

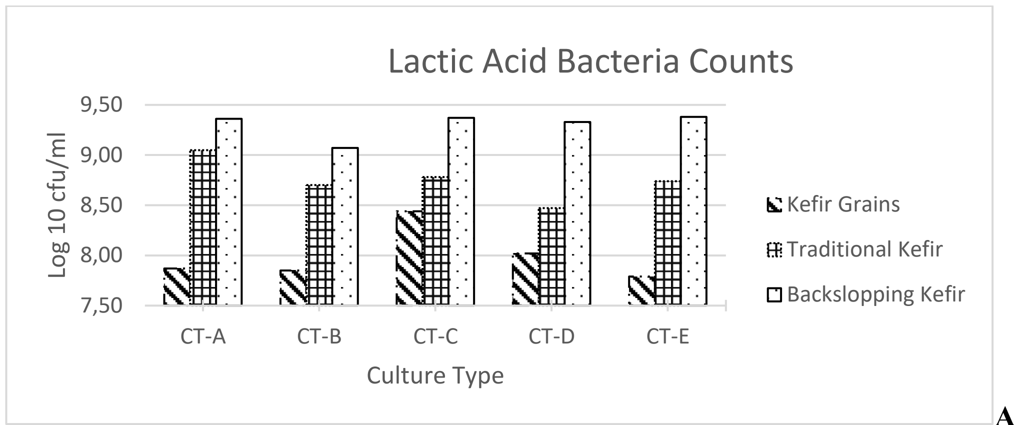
	LAB (log_{10} cfu/mL)	**AAB (log_{10} cfu/mL)**	**Yeasts (log_{10} cfu/mL)**
KD-CT A	8.76^{AB}	8.63^{AB}	6.19^{B}
KD-CT B	8.54^{B}	8.62^{AB}	6.81^{A}
KD-CT C	8.86^{A}	8.81^{A}	6.96^{A}
KD-CT D	8.61^{B}	8.59^{B}	6.75^{A}
KD-CT E	8.64^{AB}	8.79^{A}	6.33^{B}

Table 7. Lactic Acid Bacteria (LAB), Acetic Acid Bacteria (AAB) and yeasts mean comparisons (HSD test) of kefir drink culture type (KD-CT). Means within the same column with different letters are different at $P \leq 0.05$. KD-CT = kefir drink culture type

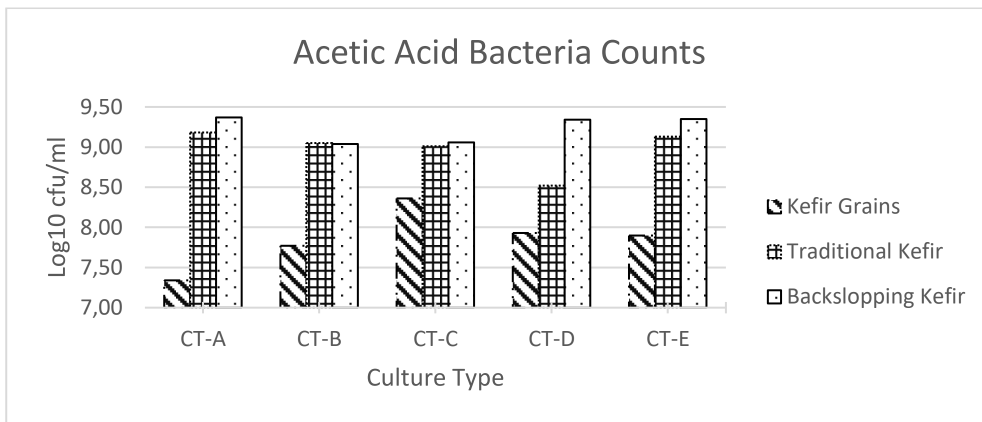
The interaction analysis revealed interesting differences in the trend of the five culture types, from the grains to the drinks. The LAB counts increased from grains to TK for all the five cultures with different rates (Figure 2A), but similar final counts were detected in BK.

Figure 2B shows that, nevertheless significant differences among kefir grains, the five cultures reached similar AAB counts in the BK.

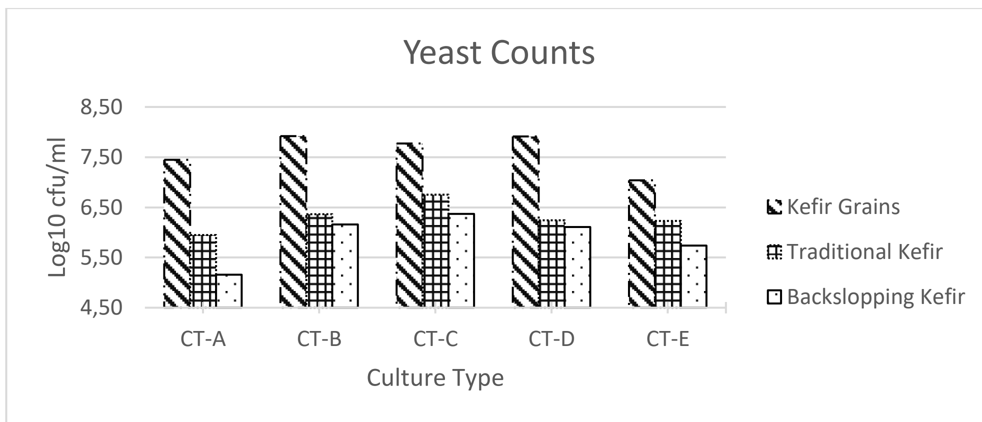
A decrease in yeast counts (Figure 2C) was observed moving from grains to TK and then to BK, but with a different trend for each culture. However, a significant difference between the TK and BK was detected only for culture type A.



A



B



C

Figure 2. KD x KD-CT interaction: Lactic Acid Bacteria (A), Acetic Acid Bacteria (B) and yeasts (C) (Log_{10} cfu/ml) in kefir grains, traditional (TK) and backslopped (BK) kefir of the 5 culture types

Beside these differences among culture types, overall the LAB and yeast counts of kefir grains in the present study (7.99 and 7.62, respectively; Table 6) were in line with those generally reported in literature that may vary from 4 to 9 log cfu/g and from 5 to 8 log cfu/g, respectively (Rea et al., 1996; Motaghi et al., 1997; Witthuhn et al., 2004; Guzel-Seydim et al., 2005; Sarkar, 2008; Kök Taş et al., 2012; Garofalo et al., 2015; Arslan, 2015; Prado et al., 2015). LAB counts increased until 8.75 log cfu/g in TK and 9.30 log cfu/g in BK reaching values very similar to those reported by Kim et al. (2018). Concerning AAB, they are usually considered as minority species within kefir grains microbiota since they are only occasionally detected with counts from 4 to 6 log cfu/g (Farnworth, 2005, Dobson et al., 2011; Guzel-Seydim et al., 2011; Garofalo et al., 2015; Rea et al., 1996), whereas in the present study AAB viable counts were higher than 7 log cfu/g (Table 6). Furthermore, AAB numbers increased in TK and BK reaching values (8.98 log cfu/g and 9.23 log cfu/g, respectively; Table 6) that were higher than those reported in literature for AAB in TK (6 log cfu/g; Irygoyen et al., 2005). Yeast counts in kefir grains were in line to the ones reported by Garofalo et al. (2015) and Guzel-Seydim et al. (2005). Furthermore, TK showed yeasts counts very similar to those reported by Guzel-Seydim et al. (2005) (6.16 log cfu/g) and in line with Arslan (2005) that reported values ranging from 3 to 6 log cfu/g. As already discussed, TK and BK yeast counts were in line to those reported by Kim et al. (2018).

Microbiota in kefir grains (KG), traditional (TK) and backslopped (BK) kefir

The bacterial diversity of the KGs, and the kefir drinks (TKs and BKs) was obtained through rRNA amplicon sequencing. The total number of paired sequences obtained from 16S rRNA sequencing reached 456269 raw reads. After data analysis, a total of 358468 reads passed the filters applied through QIIME, with a median value of 21362 ± 13.996 reads/sample, and a mean sequence length of 465 bp. The rarefaction analysis and Good's coverage, expressed as a median percentage (98%),

indicated satisfactory coverage for all samples. Alpha-diversity index showed a higher level of complexity and highest number of OTUs in TK and BK samples if compared with KGs (FDR < 0.05). No significant difference was observed between TKs and BKs for all indexes (Figure 3).

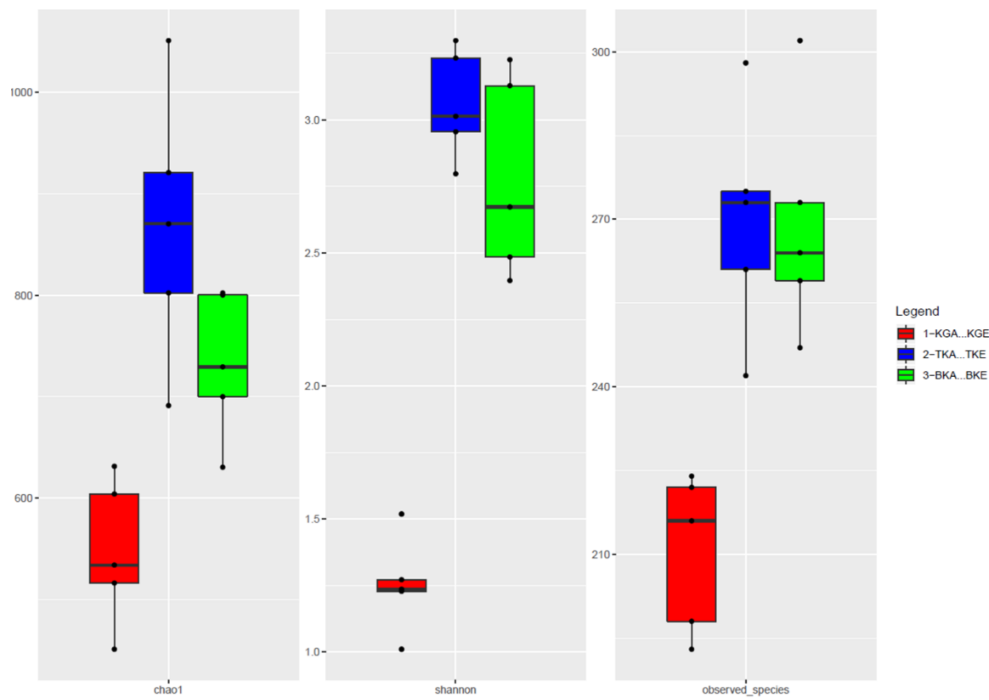


Figure 3. Boxplots to describe α-diversity measures of microbiota of KG, kefir grain (red bars), TK, traditional kefir (blue bars) and BK, backslopped kefir (green bars). Individual points and brackets represent the richness estimate and the theoretical standard error range, respectively

Adonis and analysis of similarity (ANOSIM) statistical tests based on Unweight UniFrac distance matrix showed significant differences among the three type of samples ($P < 0.001$). Differences between samples were further demonstrated by principal-coordinate analysis (PCoA) based on the Unweight UniFrac distance matrix (Figure 4). The PCoA clearly showed a separation between the three types of samples (KGs vs. BKs vs. TKs).

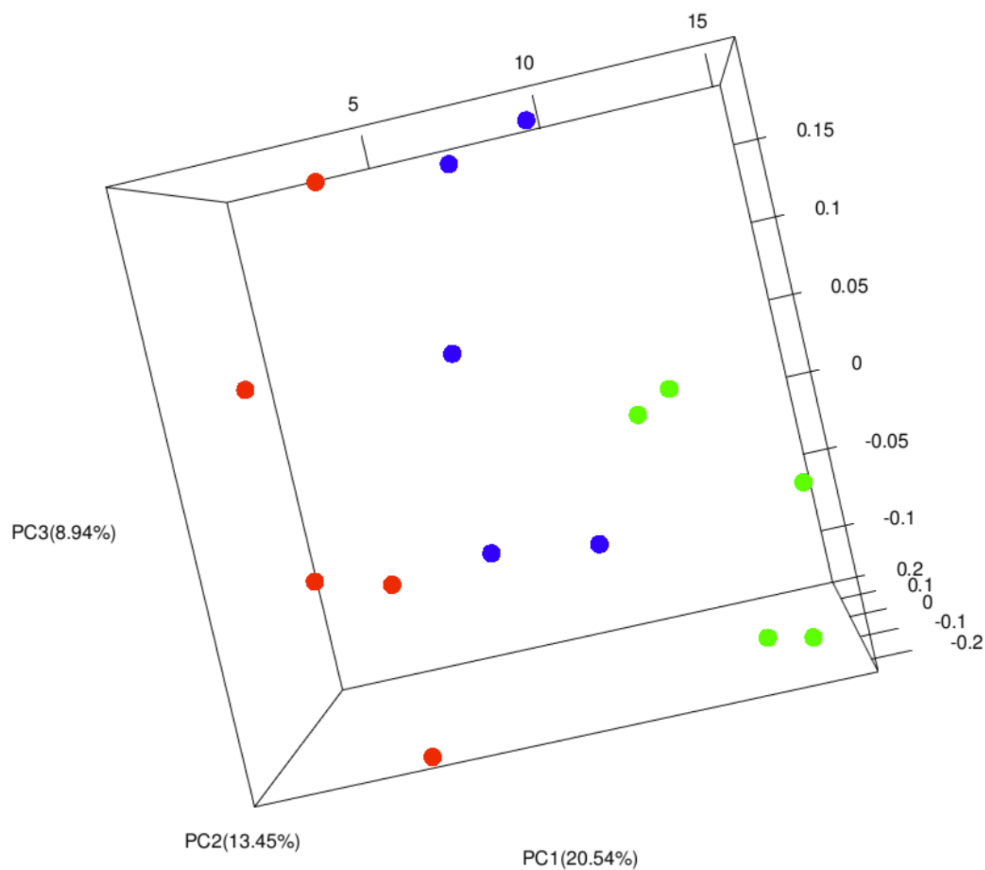


Figure 4. Principal coordinates analysis of Unweighted UniFrac distances for 16S rRNA gene sequence data. Samples are color-coded as follow: KG, kefir grain (red dots), TK, traditional kefir (blue dots), and BK, backslopped kefir (green dots).

As shown in Figure 5 we observed a very simple microbiota composition dominated by the presence of *L. kefiranofaciens*: median value of 92.72, 42.37 and 22.17 % of the relative abundance in kefir grains, TKs and BKs respectively; *Acetobacter syzygii* 4.22, 27.09 and 18.76% of the relative abundance in kefir grains, TKs and BKs respectively; and *L. lactis* 0.83, 16.75 and 50.06 of the relative abundance in kefir grains, TKs and BKs, respectively.

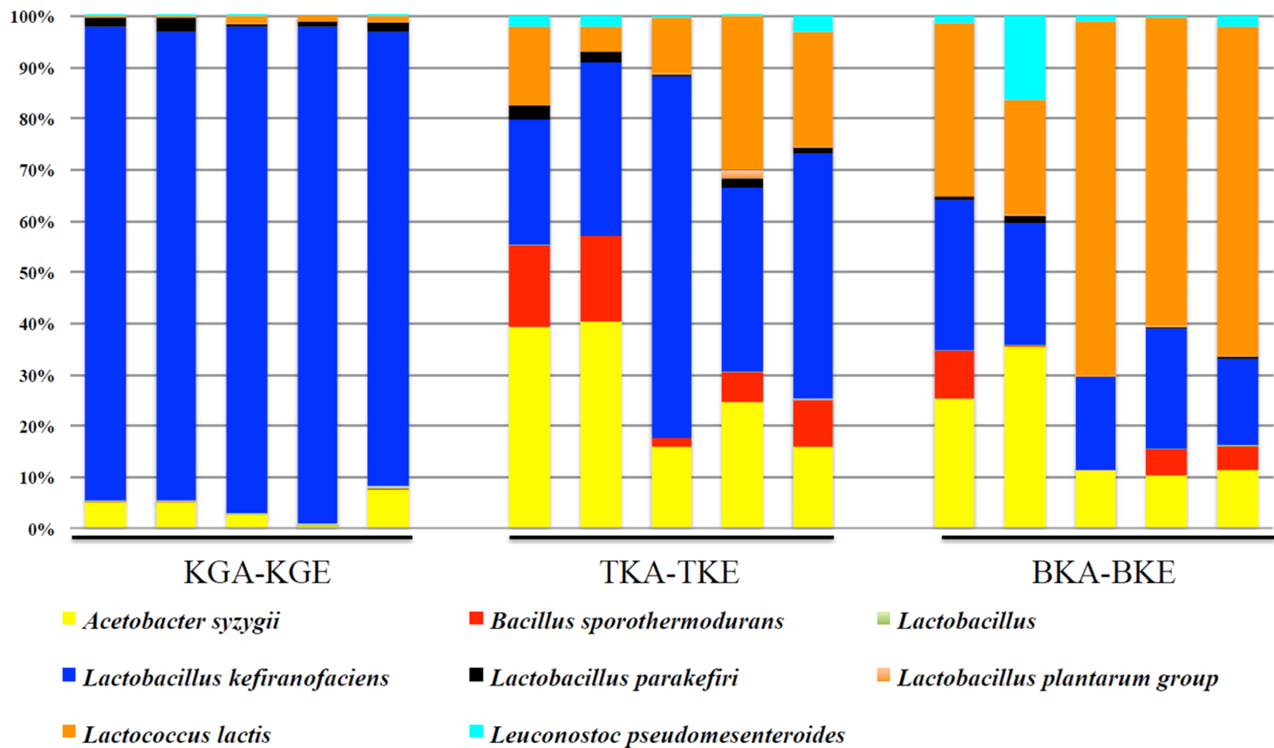


Figure 5. Incidence of the major taxonomic groups detected by 16S rRNA sequencing. Only OTUs with an incidence above 0.2% in at least two samples are shown. KGA-KGE, kefir grain; TKA-TKE, traditional kefir; BKA-BKE, backslopped kefir.

In addition, we observed the presence of minor OTUs such as in *Leuconostoc pseudomesenteroides* especially in BKs (10% median value), *Bacillus sporothermodurans* (10% median value) in TKs samples, *Lactobacillus parakefiri* in both kefir grains and TKs (2%) and then reaching less than 1% in BKs and few LAB spread across samples.

Regarding the main OTUs we observed (Figure 6) that *Lb. kefiranofaciens* and *Lactobacillus* spp. were associated with kefir grain samples (FDR < 0.05), *A. syzygii* and *B. sporothermodurans* with TK samples, while *Lc. lactis* and *Leuc. pseudomesenteroides* with BK (FDR < 0.05).

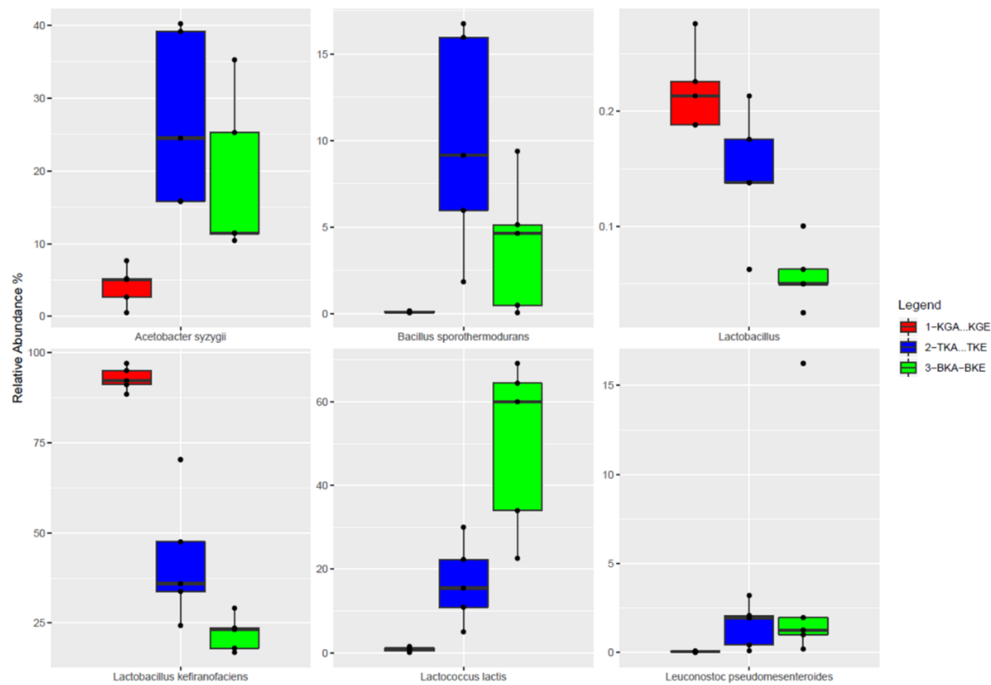


Figure 6. Boxplots showing the relative abundance at of the OTUs differentially abundant based on Pairwise Wilcoxon test (FDR ≤0.05) in kefir grain (KG), traditional kefir (TK) and backslopped kefir (BK).

Lactobacilli are generally dominating within the kefir grains microbiota (Leite et al., 2012; Garofalo et al., 2015; Kotova et al., 2016; Dertli and Çon, 2017; Bengoa et al., 2018). *Lb. kefiranofaciens*, dominated the kefir grains under study as already reported for kefir grains collected worldwide (Plessas et al., 2017; Bengoa et al., 2018). *Lb. kefiranofaciens* is a homofermentative species considered a key species within kefir grains microbiota since it is mainly involved in the grain formation and production of kefiran (Wang et al., 2012; Hamet et al., 2013; Bengoa et al., 2018). Relative abundance of *Lb. kefiranofaciens* was reduced in the kefir fermented drinks (TK and BK) where other different species prevailed. The species *A. syzygii* found in very low amount in the kefir grains becoming consistent in kefir drinks, mainly in TKs. This interesting species, found the first time in Brazilian kefir grain by da Miguel et al. (2010), was found capable to bind mycotoxins in milk and reduce their gastrointestinal absorption (Taheur et al., 2017). Overall, among AAB, *Acetobacter* is indicated as the most abundant genera within kefir grains (Bengoa et al., 2018). The species *Lc. lactis* and *Leuc. pseudomesenteroides* were found to be significantly prevalent in the kefir drinks

more than kefir grains, as already found in other studies (Simova et al., 2002; Kesmen and Kacmaz, 2011; Leite et al., 2013 b; Korsak et al., 2015; Kotova et al., 2016; Gao and Zhang, 2019). In detail, Gao and Zhang (2019) reported the same bacterial shift found in the present study from *Lb. kefiranofaciens* which was dominant among kefir grains from Tibet, China to *Lc. lactis* and *Leuc. pseudomesenteroides* that increased their relative abundance in TK. These differences among kefir grains microbiota and kefir drinks microbiota have been explained by the different distribution of microorganisms on the grains. It is indeed reported that long bacilli (rod-shaped bacilli o lactobacilli) are generally distributed on the inner layers of the grains while cocci, as also observed for the yeasts, are generally localized on the outer portion of the grains and are characterized by a weak adhesion to the grains (Rea et al., 1996; Guzel-Seydim et al., 2005; Jianzhong et al., 2009; Magalhães et al., 2010; 2011; Gao et al., 2012; Wang et al., 2012). Therefore, cocci may easily fall within milk and, thanks to their strong ability to growth in milk, they became dominant in kefir drinks (Gao and Zhang, 2019). In detail, Gao and Zhang (2019) indicated that *Leuc. pseudomesenteroides* is able to growth in association with lactococci also playing an important role in producing aromatic and flavoring compounds within the final product.

The mycobiota characterization was obtained by sequencing the D1 domain of the 26S genes and a total of 1378677 raw sequence were obtained and 1369182 pass the quality filtering with a median length value of 389 bp. Rarefaction analysis and Good's coverage display a satisfactory coverage (99%) in all the samples. Alpha-diversity index (Figure 7) showed a higher level of complexity in TK and BK samples if compared with kefir grains (FDR < 0.05). No significant difference in term of number of observed species between kefir grain and TKs, but were significantly lower if compared with BKs.

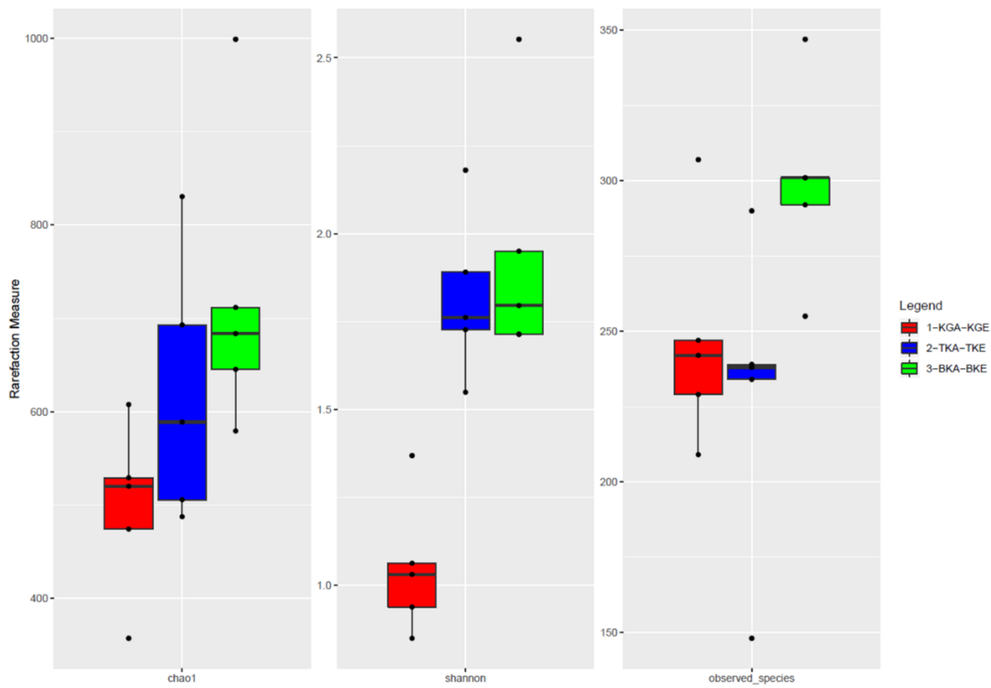


Figure 7. Boxplots to describe α-diversity measures of mycobiota of kefir grain, KG (red bars), traditional kefir, TK (blue bars) and backslopped kefir, BK (green bars). Individual points and brackets represent the richness estimate and the theoretical standard error range, respectively

Significative difference among the three type of samples (ANOSIM $P<0.001$) was observed through PCA based on OTU table abundance (Figure 8) where kefir grain samples were well separated and a slight separation was observed between TKs and BKs.

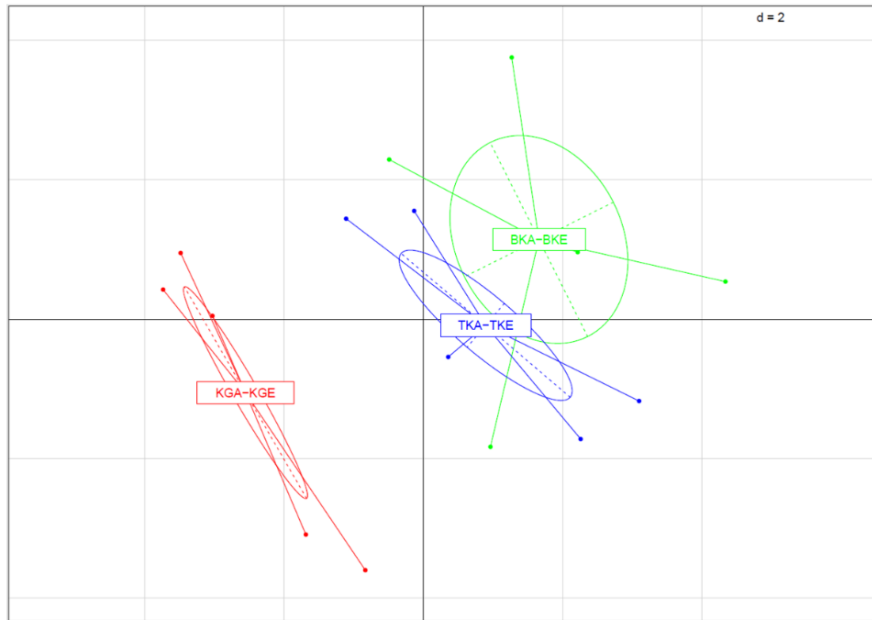


Figure 8. PCA based on the OTU abundance of 26S datasets as a function of the samples type. The first component (horizontal) accounts for the 40.66% of the variance and the second component (vertical) accounts for the 22.53 %.

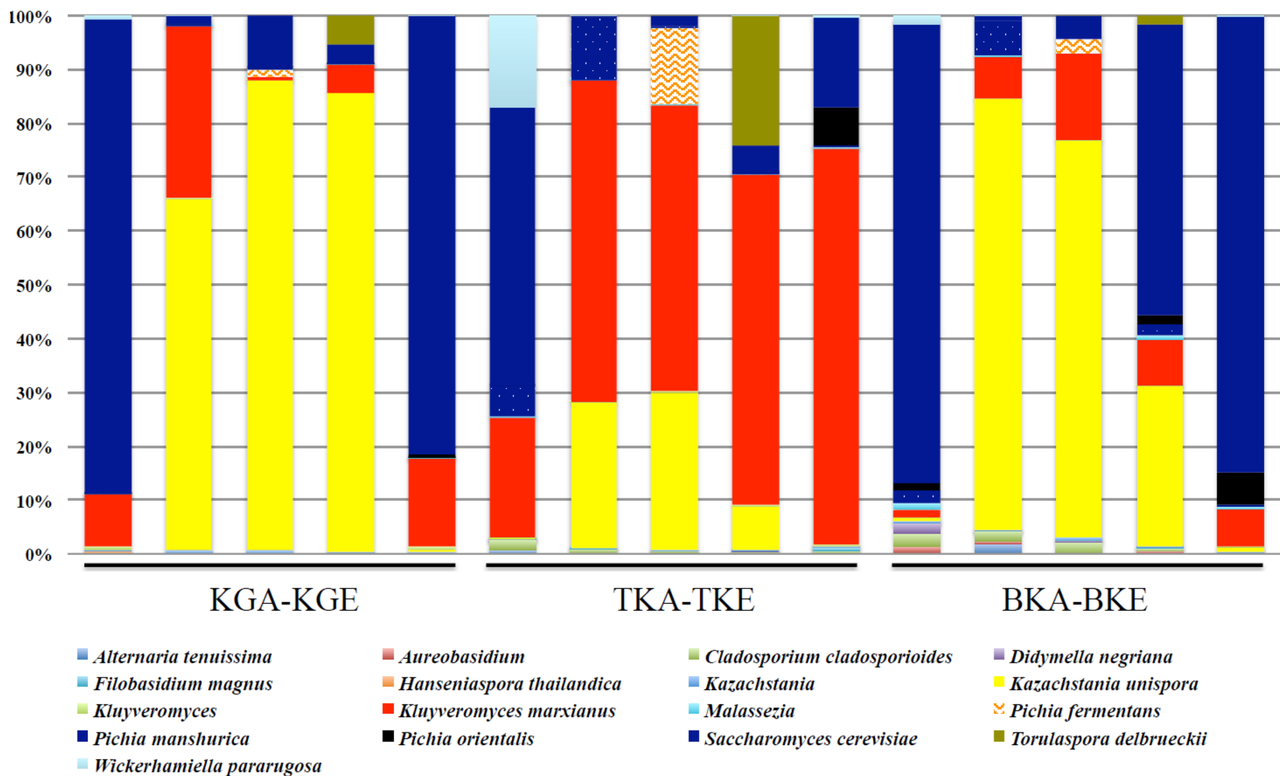


Figure 9. Incidence of the major taxonomic groups detected by 26S rRNA sequencing. Only OTUs with an incidence above 0.2% in at least two samples are shown.

The core mycobiota was composed mainly by the presence of *Kazachstania unispora* (47.40, 12.69 and 32.31% in kefir grains, TKs and BKs, respectively as a median value of the relative abundance), *Saccharomyces cerevisiae* (36.55, 14.78 and 39.24%), and *Kluyveromyces marxianus* (12.69, 52.78, 7.04%) (Figure 9). All these yeast species were mainly isolated from kefir grains and beverages worldwide (Prado et al., 2015; Plessas et al., 2017; Bengoa et al., 2018). In particular, B, C and D kefir grains were characterized by the prevalence of *K. unispora* species (also called *Saccharomyces unisporus*), whereas the kefir grain A and D were characterized by the dominance of *S. cerevisiae*. In all the kefir grains was detected the presence of *Kl. marxianus* that became more consistent in TKs. The mycobiota of BK samples remained almost similar to that of kefir grain samples. *Kl. marxianus* is a lactose positive yeast able to metabolize lactose of the milk as carbon source and therefore, this yeast species is able to grow in milk, thus ensuring the formation of ethanol, CO_2 and the typical yeasty flavor of kefir (Vardjan et al., 2013). On the opposite, *S. cerevisiae* and *K. unispora* are non-lactose fermenting yeasts that may multiply in kefir grains and milk by using galactose and glucose released by lactose hydrolysis caused by other bacteria and yeast species as *Kl. marxianus* inhabiting within kefir grains and in fermented milk (Diosma et al., 2013; Garofalo et al., 2015).

It should be also noted that TK samples display a higher abundance of *Torulaspora delbrueckii*, *Pichia fermentans*, *Pichia manshurica* and *Pichia orientalis* if compared with kefir grains and BK samples. *T. delbrueckii* has been already found in kefir grains from Slovenia (Vardjan et al., 2013), while *P. fermentans* was identified in Taiwanese kefir (Wang et al., 2008 b). To the authors knowledge *Pichia manshurica* and *Pichia orientalis* have never been detected among kefir grains and kefir drinks.

By comparing the relative abundance of the mycobiota it was observed that several minor OTUs characterized the different samples (Figure 10; FDR < 0.05). In particular, it was observed that *Alternaria tenuissima*, *Cladosporium cladosporioides* and *Malassezia* were associated with BK samples, while *Kazachstania* was associated with kefir grains and *Kl. marxianus* with TKs (Figure

10). To the authors knowledge the filamentous fungi *A. tenuissima* and *Cl. cladosporioides* have never been isolated from kefir grains and kefir drinks while the genera *Malassezia* was already detected with low abundance and in just one kefir samples by Marsh et al. (2013).

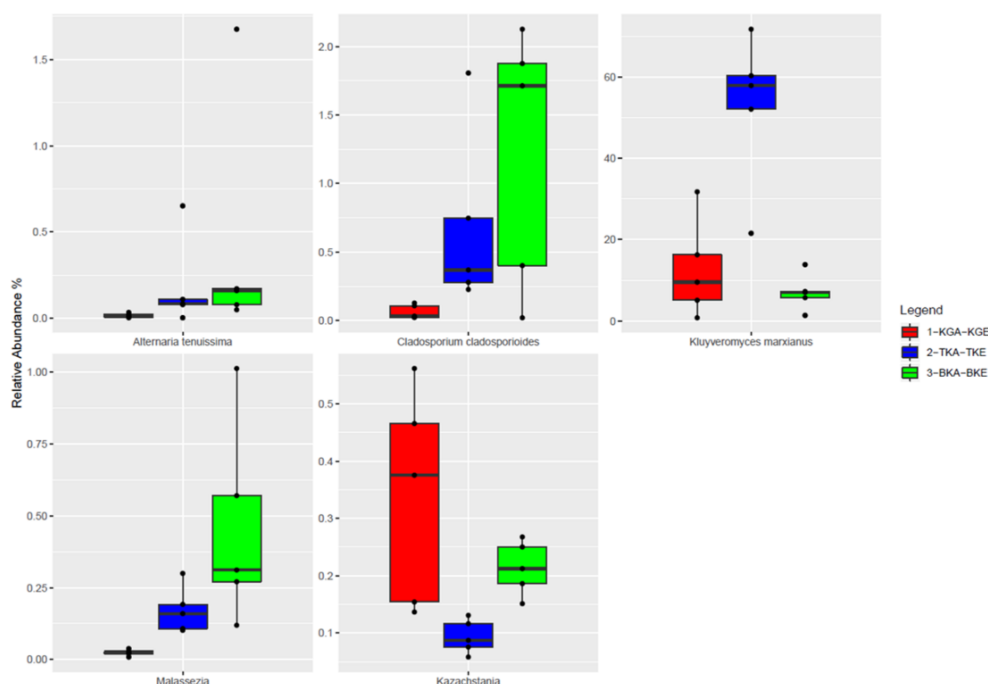


Figure 10. Boxplots showing the relative abundance at of the OTUs differentially abundant based on Pairwise Wilcoxon test (FDR ≤0.05) in kefir grain (KG), traditional kefir (TK) and backslopped kefir (BK).

Volatile organic compounds (VOCs) in traditional (TK) and backslopped (BK) kefir

The SPME-GC/MS analysis allowed to individuate more than 60 volatile components in the TK and BK samples. Peaks with area <1% of the total peak areas and with no significant differences (ANOVA, Tukey's HSD test) in the different conditions were discarded from further statistical and graphical analyses. Table 8 shows the thirty-one volatile components that mainly ($P < 0.05$) (ANOVA) characterized the samples. The most characteristic volatile compounds belonged to four classes such as ketones, esters and acetates, alcohols and acids.

Taking into the account the volatilome composition among the TK and BK samples, it was clearly observed a separation between those type of samples based on the VOCs composition highlighting that the different methods used to produce the kefir beverages (TK and BK) significantly influenced the final flavor of products. Similar to diversity of microbial flora of the TK and BK kefir there was a high level of diversity in the volatile components.

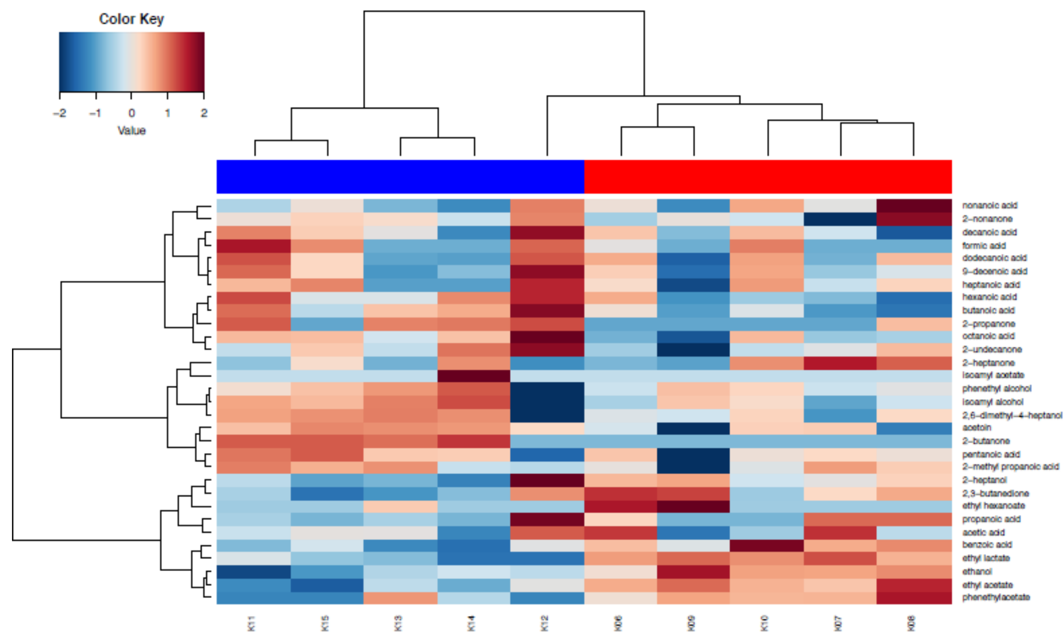


Figure 11. Hierarchical clustering between samples and VOCs. Rows and columns are clustered by Ward linkage hierarchical. The intensity of the colors represents the degree of correlation between the samples and VOCs. The upper bar is color coded according to the sample's type [traditional kefir (TK): red, backslopped kefir (BK): blue].

RI	Compounds	Traditional kefir					Backslopped kefir					Odor*
		A	B	C	D	E	A	B	C	D	E	
	Ketons											
774	2-propanone	nd	nd	0.1±0.0	nd	nd	0.5±0.08	0.3±0.05	0.2±0.01	0.2±0.01	nd	solvent, ethereal
924	2-butanone	nd	nd	nd	nd	nd	0.1±0.01	nd	nd	0.1±0.01	0.1±0.01	Buttery, yogurt-like aroma
1008	2,3-butanedione	1.9±0.2	1.1±0.2	1.4±0.2	1.9±0.3	0.7±0.01	0.7±0.08	1.5±0.01	0.6±0.1	0.7±0.1	0.5±0.06	buttery, sweet, creamy
1158	2-heptanone	1.1±0.1	4.4±0.4	3.4±0.1	1.0±0.1	2.8±0.1	1.2±0.1	0.9±0.1	1.0±0.1	2.8±0.1	1.9±0.2	cheesy
1275	acetoin	8.6±0.4	9.8±1.3	6.4±0.5	5.1±0.8	9.7±0.4	10.1±0.5	9.5±1.3	11.0±1.3	10.9±0.6	11.2±0.5	buttery, creamy, dairy
1390	2-nonanone	1.3±0.06	1.0±0.1	2.0±0.2	1.5±0.1	1.4±0.1	1.7±0.2	1.6±0.2	1.4±0.01	1.4±0.01	1.5±0.2	cheesy, sweet
1598	2-undecanone	0.4±0.0	0.5±0.02	0.5±0.04	0.3±0.01	0.4±0.01	0.4±0.05	0.6±0.02	0.4±0.05	0.5±0.03	0.5±0.1	fruity, creamy
	Esters and acetates											
910	ethyl acetate	1.0±0.1	0.9±0.1	2.2±0.2	1.5±0.1	1.0±0.1	0.2±0.01	0.6±0.02	0.4±0.05	0.3±0.01	0.1±0.01	fruity, sweet
1116	isoamyl acetate	nd	nd	nd	nd	nd	nd	nd	nd	0.1±0.01	nd	fruity, banana
1188	ethyl hexanoate	0.2±0.02	nd	nd	0.2±0.02	nd	nd	nd	0.04±0.00	nd	nd	fruity, waxy, green banana
1298	ethyl lactate	0.5±0.03	0.9±0.1	0.4±0.05	0.8±0.06	0.6±0.06	0.1±0.01	nd	0.05±0.01	nd	0.06±0.01	fruity, buttery, butterscotch
1799	phenethylacetate	0.3±0.04	0.6±0.04	3.6±0.04	0.8±0.03	0.6±0.06	nd	nd	0.9±0.05	0.1±0.01	nd	fruity, sweet, honey
	Alcohols											
957	Ethanol	9.7±1.2	15.5±0.5	17.9±1.4	35.9±4.4	15.8±2.5	1.9±0.2	6.4±1.0	5.9±0.12	7.2±0.1	3.5±0.5	solvent, ethereal,
1210	isoamyl alcohol	4.5±0.5	3.4±0.5	5.2±0.4	7.6±1.1	6.7±0.6	8.9±1.1	1.7±0.2	10.3±1.0	12.5±0.4	8.1±0.2	fermented
1280	2-heptanol	0.7±0.05	0.4±0.05	0.6±0.08	0.7±0.1	0.4±0.03	0.3±0.05	2.2±0.3	0.3±0.03	0.2±0.02	0.2±0.02	fruity, fresh lemon
1460	2,6-dimethyl-4-heptanol	2.2±0.3	0.3±0.04	4.0±0.2	1.7±0.16	4.3±0.5	9.3±1.1	nd	14.5±1.7	11.8±1.5	11.5±0.2	fermented yeasty
1925	phenethyl alcohol	1.6±0.2	1.6±0.1	1.9±0.1	2.9±0.1	2.5±0.1	2.2±0.3	0.2±0.02	3.7±0.4	5.0±0.2	2.9±0.2	floral rose
	Acids											
1446	acetic acid	33.9±0.8	34.3±1.1	21.2±3.1	16.0±0.1	20.1±1.7	21.7±0.5	31.9±3.6	23.2±3.5	16.7±1.1	23.3±2.9	acidic, pungent sour vinegar
1474	formic acid	0.1±0.0	nd	nd	nd	0.5±0.06	1.6±0.1	0.7±0.05	nd	nd	0.5±0.04	acidic, pungent vinegar
1480	propanoic acid	0.1±0.01	0.1±0.01	0.1±0.01	0.05±0.001	0.05±0.001	0.06±0.001	0.2±0.02	0.06±0.001	0.05±0.001	0.05±0.001	acidic, pungent, dairy-like
1580	isobutanoic acid	0.3±0.01	0.4±0.02	0.3±0.05	0.1±0.01	0.3±0.02	0.4±0.05	0.3±0.01	0.4±0.01	0.3±0.02	0.4±0.03	acidic, dairy creamy
1605	butanoic acid	1.3±0.06	0.9±0.04	0.8±0.02	0.9±0.08	1.1±0.05	1.5±0.07	1.8±0.2	1.3±0.07	1.3±0.1	1.0±0.05	cheesy, dairy-like
1684	pentanoic acid	1.3±0.1	1.1±0.1	1.0±0.08	0.3±0.01	1.0±0.02	1.8±0.1	0.4±0.01	1.2±0.1	1.2±0.1	2.0±0.01	cheesy, dairy milk cheesy
1843	hexanoic acid	7.6±0.9	6.1±0.3	5.5±0.04	5.8±0.08	6.3±0.3	8.4±0.7	8.7±0.7	6.7±0.6	7.8±0.8	6.7±0.1	sweet cheese, yogurt-like aroma
1978	heptanoic acid	0.3±0.03	0.2±0.01	0.3±0.01	0.1±0.01	0.3±0.02	0.3±0.03	0.5±0.04	0.2±0.01	0.2±0.01	0.4±0.04	cheesy sweat
2057	octanoic acid	6.4±1.0	6.6±0.1	6.7±0.6	5.5±0.3	8.0±1.2	8.0±1.0	10.4±0.5	7.1±0.4	7.9±0.2	8.0±0.2	fatty, cheesy
2159	nonanoic acid	0.3±0.03	0.3±0.01	0.9±0.1	0.1±0.001	0.4±0.04	0.2±0.02	0.5±0.03	0.2±0.02	0.12±0.01	0.3±0.02	fatty, cheese, dairy
2263	decanoic acid	3.3±0.5	2.6±0.3	1.5±0.2	2.1±0.04	3.4±0.3	4.0±0.4	5.5±0.8	2.8±0.03	1.8±0.2	3.2±0.2	fatty, unplesant rancid
2340	9-decenoic acid	0.3±0.03	0.2±0.02	0.3±0.03	0.2±0.02	0.4±0.05	0.5±0.2	0.6±0.06	0.2±0.02	0.2±0.02	0.3±0.03	fatty, waxy
2430	benzoic acid	3.4±0.3	3.4±0.3	3.6±0.2	3.0±0.1	4.3±0.06	2.6±0.3	3.0±0.1	2.5±0.2	2.4±0.3	3.0±0.4	fruity, alcholic
2470	dodecanoic acid	0.8±0.05	0.5±0.02	0.8±0.1	0.3±0.02	0.9±0.1	1.0±0.1	1.1±0.1	0.4±0.01	0.4±0.01	0.7±0.1	fatty, coconut

RI = Retention Index. identification by comparison with RI database.
nd = not detected
Results are expressed as RAP= Relative Peak Area (Area Peak Compound/Area Peak Internal Standard) 100.
* Based on flavornet (www.flavornet.org) and pherobase (www.pherobase.com) online databases.

Table 8. Volatile organic compounds (VOCs) identified by solid phase microextraction/gas chromatography-mass spectrometry in five traditional and backslopped kefir

In detail, the hierarchical cluster analysis clearly showed this separation (Figure 11). TKs were mainly characterized by the presence of short chain fatty acid (es. acetic, benzoic, propanoic acids), different acetates such as ethyl acetate, ethyl lactate, phenethylacetate, and ketons (2,3−butanedione, 2-heptanone) and ethanol. In addition, isoamyl alcohol, 2,6-dimethyl-4-heptanol and phenethyl alcohol were characteristic of TK samples. On the other hands BK samples showed the predominance of medium and long chain fatty acid (pentanoic, butanoic, hexanoic, heptanoic, octanoic and decanoic acids) and ketones compounds (acetoin and 2-nonanone).

Among carboxylic acids, acetic acid was the most representative one in all the fermented drinks (Table 8).

As recently reviewed by Mota-Gutierrez et al. (2019) VOCs are organic compounds naturally produced by microorganisms during fermentations as secondary metabolites that may also provide health benefits to consumer beside sensory features. In particular, among the VOCs detected within kefir drinks, phenethyl alcohol (or 2-phenylethanol) that confers a floreal rose like odor, and phenethylacetate that is responsible of fruity, sweet and honey flavor have been demonstrated to have an antimicrobial activity turn in particular to inhibit the growth of Gram negative bacteria and filamentous fungi (Mota-Gutierrez et al., 2019). Moreover, several studies have been demonstrated that phenethyl alcohol can be used to counteract the olfactory dysfunction due to multiple etiologies (Mota-Gutierrez et al., 2019). The synthesis of phenethyl alcohol is a pathway that involves the transamination of the amino acid L-phenylalanine to phenylpyruvate, the decarboxylation to phenylacetaldehyde and the reduction to alcohol carried out by the yeasts' metabolism mainly by *Kl. marxianus*, *Pichia anomala*, *Pichia farinosa*, *Pichia kudriavzevii*, *S. cerevisiae* and *Wickerhamomyces anomalus* that have been found involved within cocoa beans fermentation (Mota-Gutierrez et al., 2019). Also, the esters phenethylacetate (or 2-phenylethyl acetate) and ethyl acetate derive from transformation of amino acids that are metabolized by yeasts species ascribed to *Kl. marxianus*, *Kluyveromyces lactis*, *Hanseniaspora uvarum*, *Hanseniaspora guilliermondii*, *Candida*

tropicalis, *Candida utilis*, *Geotricum candidum*, *Pichia anomala*, *Pichia farinosa*, *Pichia kudriavzevii*, *S. cerevisiae* and *Wickerhamomyces anomalus* already found as important producers of these desirable compounds during cocoa beans fermentation (Mota-Gutierrez et al., 2019). In detail, 2-phenylethyl acetate is well-known for its antimicrobial activity and it is obtained by metabolization of phenylalaline and/or phenylpyruvic acids, while ethyl acetate is obtained through esterification of leucine, isoleucine or valine and a natural aliphatic alcohol (Mota-Gutierrez et al., 2019).

By comparing the presence of the VOCs among the samples we observed the predominance (FDR < 0.05) in TKs of ethyl acetate, ethyl lactate, benzoic acid and ethanol. The presence of ethanol undoubtedly gives to the final products a desired flavor of a light alcoholic beverage and higher alcohol content may be associated with a slight yeasty flavor (Guzel-Seydim et al., 2000 a). While BK samples displayed the presence of 2-propanone, 2-butanone, acetoin, butanoic and hexanoic acids (Figure 12; FDR < 0.05) that are determinant volatile components since they are associated with yogurt-like aroma or cheese sweet flavor. Acetoin is also reported by Guzel-Seydim et al. (2000 a, b) as one of the major end products of microbial fermentation characterizing kefir aroma.

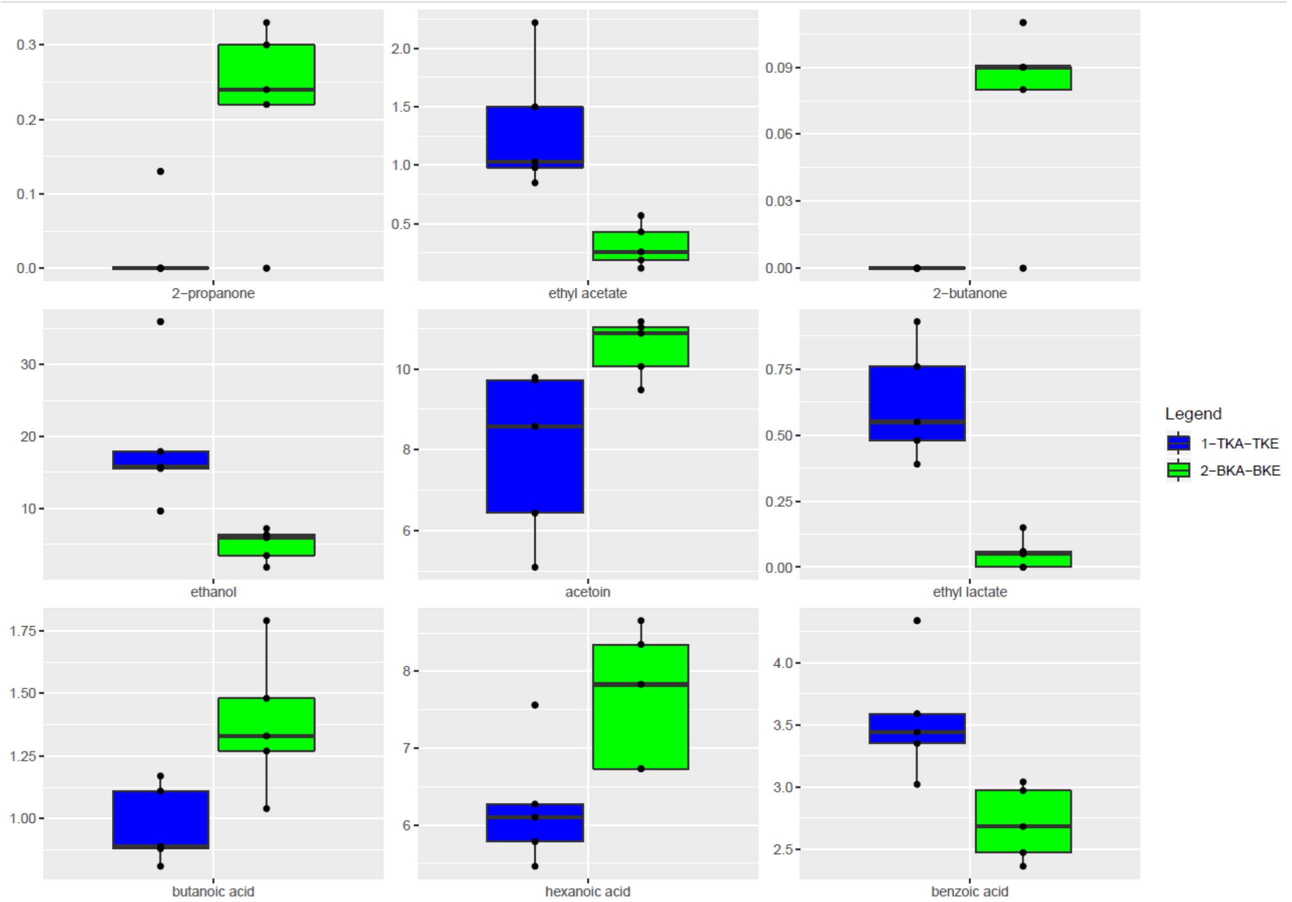


Figure 12. Boxplots showing the relative abundance at of the VOCs differentially abundant based on Pairwise Wilcoxon test (FDR ≤0.05) in traditional kefir (blue bars) and backslopped kefir (green bars).

By plotting the correlation between VOCs and microbiota (Figure 13; FDR < 0.05) it was observed that *A. syzygii* displayed a significant positive correlation with 2,3−butanedione and 2−heptanol while *B. sporothermodurans* with ethyl lactate and both these bacteria were associated with TK samples. More in detail, the production of secondary alcohols as 2-heptanol can be achieved from 2-heptanone (Mota-Gutierrez et al., 2019).

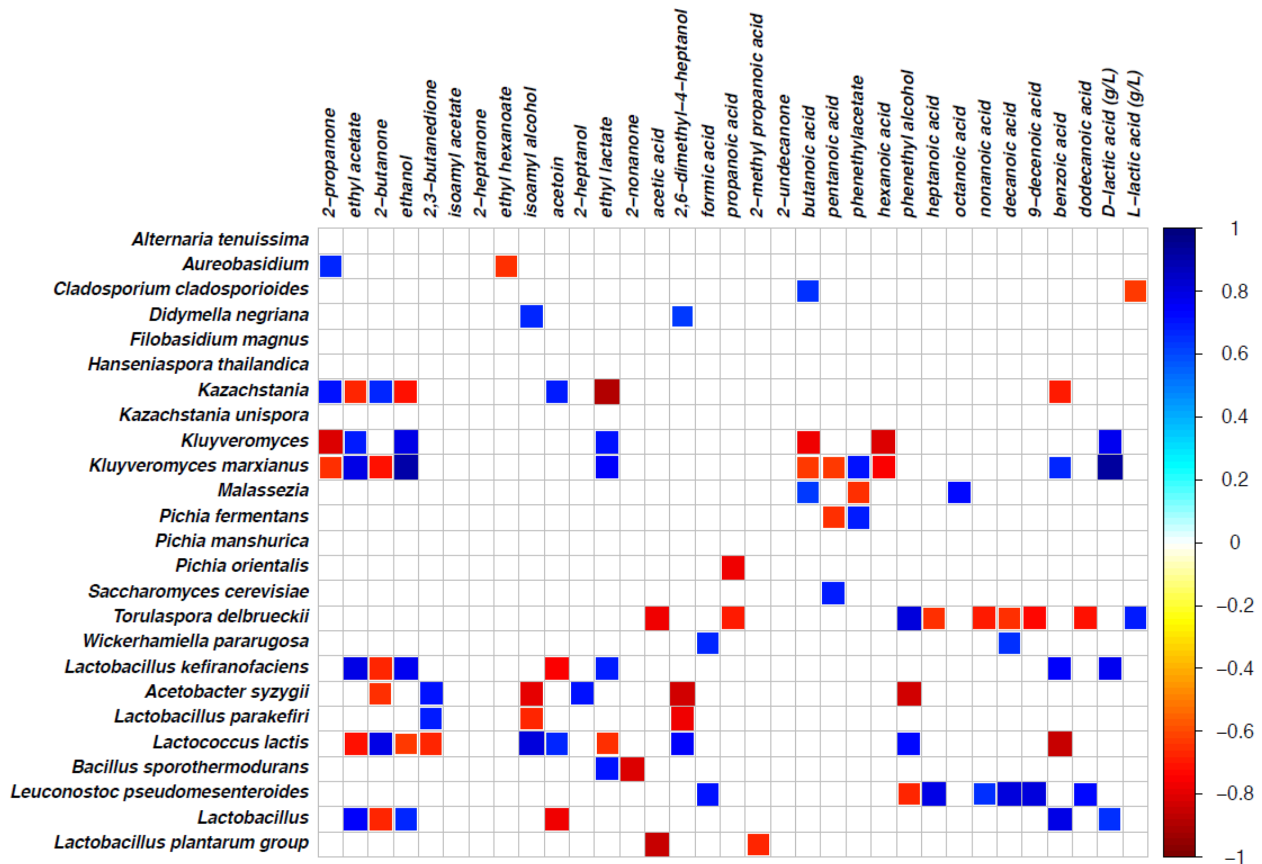


Figure 13. Correlation between the abundance of VOCs (mg/kg) and OTUs occurring at 0.2% in at least 2 samples. The intensity of the colors represents the degree of correlation between the samples and VOCs as measured by the Spearman's correlations.

The main OTUs of the BK samples *Lc. lactis* was found correlated with 2−butanone, acetoin, isoamyl alcohol, 2,6−dimethyl−4−heptanol and phenethyl alcohol while *Leuc. pseudomesenteroides* correlated with formic, heptanoic, octanoic, nonanoic, decanoic and dodecanoic acid (Figure 13; FDR < 0.05). *Lb. kefiranofaciens*, associated with TKs as showed in Figure 6, was highly correlated with ethyl acetate, ethanol, ethyl lactate and benzoic acid. Few correlations were observed between VOCs and mycobiota; however, it should be noteworthy the correlation between *Kl. marxianus*, the more abundant yeast in TKs samples, with ethyl acetate, ethanol, ethyl lactate, benzoic acid and phenethylacetate, volatile compounds strongly characterizing TK samples (Figure 13). Additionally,

among yeasts, *S. cerevisiae* resulted associated with pentanoic acid and *T. delbrueckii* with phenethyl alcohol (Figure 13; FDR<0.05).

Regarding the production of lactic acid, a high correlation was found between D-lactic acid and *Lb. kefiranofaciens*, *Kl. marxianus*, *Kluyveromyces* spp. and *Lactobacillus* spp. In details *Lactobacillus* spp. and *Lb. kefiranofaciens* were highly associated to the TKs whereas the amount of D-lactic acid (median value 5.27 g/L) was always higher respect to the BK samples (median value 1.68 g/L). On the other hand, a high correlation was found between L-lactic acid and *T. delbrueckii*. BKs, differently from TK samples, were characterized by high L-lactic acid (4.41 g/L, median value) and low D-lactic acid (2.61 g/L, median value) highlighting that backslopping method could move the ratio of two steroisomeric forms in favour of L-(+)-lactate.

The first two principal components (PC) explained the 70.7% of the total variance. Samples produced with traditional method (TKs) were clearly separated from samples obtained by backslopping (BKs) on the basis of the odors. TK samples were mainly correlated to alcoholic (solvent), fruity, fatty and acid odors whereas all the BK samples, with the exception of BKB were correlated to cheese, buttery, floreal and fermented odors.

The results related to kefir VOCs composition provide evidence indicating that different processing conditions, traditional vs backslopping methods, influence both the microbial composition and the volatile compounds pattern, impacting on the quality characteristics of the final products as confirmed by the Principal Component Analysis (PCA) (Figure 14).

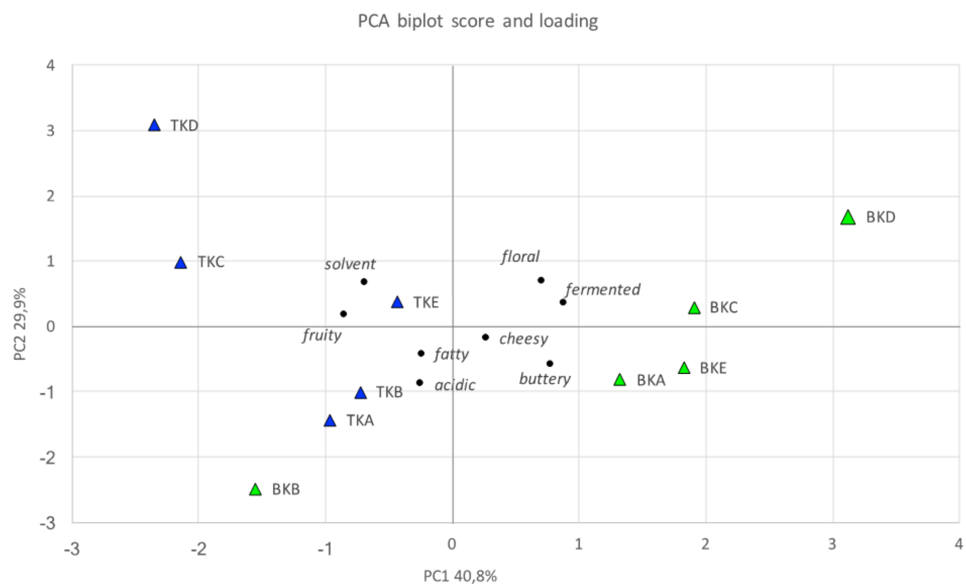


Figure 14. Score and loading plot of first and second principal components after principal-component analysis based on odors that mainly ($P < 0.05$) differentiated the traditional kefir (TKA-TKE) and the backslopped kefir (BKA-BKE). Volatile organic compounds used in PCA are listed in Table 8

Conclusions

The kefir grains under study collected from Sarajevo (Bosnia and Herzegovina) were found dominated by lactobacilli and in particular, by *Lb. kefiranofaciens*. Then a shift in the bacterial species toward *A. syzygii*, *Lc. lactis* and *Leuc. pseudomesenteroides* from kefir grains to traditional kefir and backslopped kefir was generally observed. Indeed, the bacterial species that were detected with higher relative abundance in kefir grains were progressively reduced in kefir drinks, while those species that were minority in grains become dominant in kefir drinks. The core mycobiota of the kefir grains under study was composed mainly by the presence of *K. unispora*, *S. cerevisiae* and *Kl. marxianus*. The mycobiota of backslopped kefir samples remained almost similar to that of kefir grain samples, but differently from LAB and AAB, the yeast counts progressively decreased from the grains to the backslopped kefir. Overall, the microbial dynamics observed may be explained by the different localization and adhesion to grains of the microbial species involved in the grain microbiota as well as to their different growth ability in milk.

Furthermore, the kefir drinks obtained with these different methods were also different in terms of physico-chemical and nutritional features as well as for volatile profiles. Overall, the backslopped kefir enhanced final drink nutritional value for most of the parameters evaluated, compared to traditional kefir coupled with a favorable pH and volatile compounds that contribute to a pleasant sensory profile. Among volatile compounds detected within kefir drinks, phenethyl alcohol and phenethylacetate, mainly associated with traditional kefir, may also provide health benefits to consumer due to their antimicrobial activity. Furthermore, phenethyl alcohol has shown to be useful against several olfactory dysfunctions. These results confirm the usefulness of potential scale-up methodology of backslopping and highlight the importance of increase the microbiological, nutritional and sensorial studies on kefir drinks obtained either with traditional or backslopped method, which can have a positive reflection on human health status.

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Chapter 4 - The potential role of essential oils as preservatives in foods and beverages against yeast spoilage

Introduction

The ubiquitous occurrence of yeasts in the environment ensures that most foods become contaminated with yeasts during some stage in the production chain. However, most of these contaminations don't result in spoilage, because the food does not present a habitat that is conductive to yeast survival and growth. Food-related yeasts can grow at acidic pH values in foodstuffs with high carbohydrate contents (Belletti et al., 2010) and are widely distributed in nature and cause the deterioration of a wide range of chilled and ambient stable edible substances such as wines, bakery products, jams and preserves, fruit products, vinegar, juices, beverages, salads, meat (Souza et al., 2007). They can also be found in dairy products such as yoghurt, kefir, and soft and fresh cheeses (Deak, 2007; Pitt and Hocking, 2009). Yeast contamination may occur at any step in the food manufacturing process such as the use of contaminated ingredients or inadequate sanitation of manufacturing facilities. Currently, due to the increase in the amount of processed foods and the longer shelf-life required by consumers, the yeasts spoilage events are growing (Van der Vossen and Hofstra, 1996). Considering that contamination of foods by spoilage yeasts frequently leads to a decreased food product shelf-life, food spoilage by yeasts is a prime issue in the food industries which significantly effects the cost and availability of the food (Tyagi et al., 2014). Moreover, the detection of yeasts in foods is difficult and time consuming due to their slow growth time. Overall, the consequences of yeast spoilage can be different, but all of them are the results of yeast growing in the food and consequent metabolic activity. These consequences include economic loss due to wasted product or product with inferior quality and value. Moreover, the yeast spoilage is related to economic loss by cost of plant clean up, product recall and product disposal. Furthermore, there is an adverse impact on company reputation and brand image. The symptoms of a food contaminated are many and varied, such as objectionable

odor and flavor, appearance (turbidity, swelling, slime formation, discoloration), taste or texture, or combination of them. Moreover, not all may be obvious to all consumers and the food may be eaten by them (Fleet, 2011). To reduce the yeast environmental contamination several interventions can be applied. Physical processes to treat the product or chemical antifungals incorporated into the product, are important to prevent or reduce yeasts spoilage and to increase the shelf-life of the final product. Although heat treatment is one of the most effective methods for yeast control in foods, it may cause undesirable changes to sensitive food products. Therefore, products that cannot be pasteurized are usually treated with weak acid preservatives, such as sorbic or benzoic acid or their salts. However, some yeasts possess genetic or acquired resistance mechanisms to weak organic acids, including the ability to degrade them or to pump out dissociated anions (Dawidowicz and Rado, 2010; Monu et al., 2016;). Exposure to sublethal concentrations of organic acids may lead to subsequent resistance development (Piper et al., 2001). Furthermore, in the last years, there has been a growing demand by consumers for products without traditional preservatives, with consequent difficulties for producers to ensure the necessary shelf-life of the foods. To this end, several studies have been carried out regarding the use of natural anti-yeast compounds as preservatives from vegetable origin in the food industry, including essential oils (EOs), to enhance the shelf-life of foods and to avoid infections, in the last years (Burt, 2004; Feng and Zheng, 2007; Souza et al., 2007; Krisch et al., 2010; Calo et al., 2015; Çoşkun et al., 2016; Monu et al., 2016). Although, EOs are typically known for their flavoring properties, they also possess antimicrobial abilities (Burt, 2004; Calo et al., 2015). Indeed, both whole plant EOs and many of the alcohols, ethers, ketones and aldehydes that are included in these oils have well documented antimicrobial properties (Burt, 2004; Calo et al., 2015). On the contrary, only a few information about the antifungal capabilities of EOs are available (Souza et al., 2007; Çoşkun et al., 2016; Krisch et al., 2010; Monu et al., 2016).

Essential oils

EOs, (also called volatile or ethereal oils) that have generally recognized as safe (GRAS) status in the US, are complex products composed by aromatic substances with a pleasant smell and a well-defined taste. EOs are aromatic oily liquids obtained from different part of the plant (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). EOs have been described by the International Organization for Standardization (ISO) as "a product obtained from the steam distillation of natural and raw plant material, through mechanical processes on the citrus epicarp or by dry distillation, after phase separation aqueous, if present, by physical processes "specifying that" EOs may undergo physical treatments, from which no significant changes in their composition result "(ISO / DIS9235, 2013). In realty, EOs can be obtained also by expression, fermentation, enfleurage and extraction, even if the steam distillation is the most common method used for producing EOs on a commercial basis (Burt, 2004). Extraction by means of liquid carbon dioxide under low temperature and high pressure produces a more natural organoleptic profile but it is much more expensive and therefore it is not generally used. The differences in organoleptic profile indicates a difference in the composition of oils obtained by solvent extraction as opposed to distillation and this may also influence any antimicrobial and antifungal properties (Moyler, 1998). Differences in chemical compositions depend also by the type of plant species, the geographical origin of the plant, the climatic conditions, the composition of the soil, the stages of the vegetative cycle and the parts of the plant used for the extraction of EOs (Angioni et al., 2006; Masotti et al., 2003). Moreover, EOs is thought to be produced by plants in response to stressors and therefore the conditions of growth may affect the yield and content of EOs (Theis and Lerdau, 2003). EOs are usually secreted as secondary metabolites that play a role in pollination and defense mechanisms against bacteria and fungi (Tajkarimi et al., 2010). EOs are in liquid colorless or yellowish form, not necessarily with the same consistency of the oils even if they are insoluble in water and soluble in organic solvents (Nazzaro et al., 2013). EOs are

volatiles and therefore need to be stored in airtight containers in the dark in order to prevent compositional changes (Burt, 2004).

Since a long time ago spices and herbs have been used for their perfume, flavor and preservative properties (Bauer et al., 2001), but the first experimental assessment of the bactericidal properties of the vapors of EOs has been reported by De la Croix in 1881 (Boyle, 1955). The antimicrobial or other biological activities of EOs are directly correlated to the presence of their bioactive volatile components (Mahmoud and Croteau, 2002). From the chemical point of view the 90-95% of EOs consist of terpene compounds (mono-, sesqui- and diterpenes), alcohols, acids, esters, epoxides, aldehydes, ketones, amines and sulfides. The non-volatile portion represents 5-10% of the total EO and mainly includes hydrocarbons, fatty acids, sterols, carotenoids, waxes, coumarin and flavonoids (Bakkali et al., 2008; Luque De Castro et al., 1999). EOs and EO components, including eugenol, carvacrol, trans-cinnamaldehyde, cinnamon bark oil, citral, and clove bud oil were inhibitory against some yeast strains, including *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, and *Z. bailii*, *in vitro* (Monu et al., 2016). Today, an estimated 3000 EOs are known, of which about 300 are commercially important, mainly destined in food (such as flavourings), perfumes (fragrances and aftershaves) and pharmaceuticals (due to their functional properties) (Bauer and Garbe, 1985; Van Welie, 1997; Van de Braak and Leijten, 1999).

Numerous EOs have been investigated and it is hard to establish an order based on anti-yeast activity, but oregano and thyme EOs are apparently among the best inhibitors (Conner and Beuchat, 1984; Kamble and Patil, 2008; Sacchetti et al., 2005; Elgayyar et al., 2001). These EOs contain the phenolic compounds carvacrol and thymol as main constituents with membrane disrupting ability. Other EOs, such as juniper, lemon, marjoram, clary sage, basil, ginger or lemon balm, containing non-phenolic main compounds have been also found to show high toxicity against yeasts (Sacchetti et al., 2005; Elgayyar et al., 2001; Tserennadmid et al., 2011; Araújo et al., 2003). According to Sachetti et al. (2005), the susceptibility of yeasts against twelve EOs in decreasing order was: *Schizosaccharomyces*

pombe > *Saccharomyces. cerevisiae* > *Yarrowia lipolytica* > *Rhodotorula glutinis*. EO components were also investigated for anti-yeast activity, in particular the MIC for the monoterpenes α-terpinene and limonene against *Kluyveromyces* and *Candida* strains was evaluated. In general, MIC of the main components of the EOs was higher than the MIC for the parent EOs, suggesting the synergistic effect of EO components. It seems that monoterpenes (α-pinene, β-pinene, α-terpinene) play, beside phenolics, a considerable role in disturbing the membrane function in yeasts (Cox et al., 2000; Parveen et al., 2004; Adegoke et al., 2000). However, the antifungal activity of EOs cannot be easily related to a single component, but to a mixture of molecules present in these oils (Ranasinghe et al., 2002). Oregano EO has demonstrated to have an anti-yeast activity that could be applied as preservatives in food industry. This EO has been tested against strains of *S. cerevisiae*, *Candida albicans*, *Candida krusei*, *Candida tropicalis*, *Pichia minuscola*, *Pichia ohmeri*, *Rhodotorula rubra and S. cerevisiae* (Souza et al., 2007). Moreover, another study has tested the antifungal activity of ten different EOs against yeast strain of *Candida zeylan*, *Candida sake*, *Candida kefyr*, *Candida lambic* and *S. cerevisiae*. Oregano and cinnamon EOs were the best effective against these yeast strain and represent a good basis for the formulation of products with potential efficacy in the control of yeast (Çoşkun et al., 2016). Overall, these studies have demonstrated the antifungal activity *in vitro*. Therefore, the next step was to test the effectiveness of EOs *in vivo* and to evaluate eventual sensory alterations due to the use of EOs.

To this end, several studies has been carried out. Lemon EO was used to extended shelf life of apple juice. Lag phases of the fermentative yeasts *S. cerevisiae* and *S. pombe* was significantly lengthened (Tserennadmid, 2011). The taste of the product was evaluated as refreshing and harmonic. In citrus based non-carbonated beverages the combination of linalool and β- pinene, together with a mild (55 °C) heat treatment, led to a lower spoilage probability. The used concentrations of the EO components (40 and 60 μl/L) had no negative impact on the flavor of the beverages, and the mild thermal treatment

below the usual temperature range (65-75 °C) reduced the energy costs of the product (Beletti et al., 2007; Beletti et al., 2010).

Overall, EOs represent an alternative to synthetic preservatives in the food industry against spoilage yeasts. Most tested yeast strain showed some (higher or lower) sensitivity to EOs or EO components, even if it is difficult to compare the results of these tests because of the diversity in methods and units used. However, considering the antifungal activity demonstrated *in vitro* and *in vivo* by EOs and EO compounds, EOs and EO compounds could be used as natural preservatives against yeasts spoilage in foods and beverages (Souza et al., 2007; Beletti et al., 2010; Tserennadmid, 2011; Monu et al., 2016). Moreover, the choice of EO and its concentration in a particular food is important because a small amount can cause sensory alterations. However, combinations of EOs with each other or with other preservation techniques can solve this problem.

In the present Ph.D. thesis two different studies have been carried out to evaluate the effectiveness of seven different EOs (lime, ginger, lemongrass, mandarin, cinnamon, orange and lemon) against different genera and species of yeasts spoilage isolated from different food matrices.

Chapter 4.1 - The antifungal activity of essential oils against *Zygosaccharomyces bailii* isolates spoilage collected from filling creams

Introduction

Zygosaccharomyces

Osmotolerant and osmophilic yeasts are the most common spoilage agents of sugar-rich foods, where a_w is the main limiting factor for the microbial growth (Deak and Beuchat, 1994). The phenomena of contamination by these spoilage yeasts are increasing, because, in the modern world, a great proportion of foods are being processed, preserved in some form, and stored or transported over long distances before consumption. Species belonging to the genus *Zygosaccharomyces* are the main cause of economic losses in the industries that produce foods and beverages rich in sugars. *Zygosaccharomyces* is a genus of yeasts belonging to the Saccharomycetaceae family. It was first described under the genus *Saccharomyces*, but later, in 1983, it was reclassified to its current name (Barnett et al., 1983). *Zygosaccharomyces* is a genus often regarded as synonymous with food spoilage.

Zygosaccharomyces and in particular *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii*, thanks to their unusual physiological characteristics that are responsible of their ability to cause spoilage. These characteristics are extreme osmotolerance, ability to ferment hexose sugars, high optimum growth temperature and resistance to weak-acid commonly used as food preservatives such as sorbic acid, benzoic acid, acetic acid and ethanol (Silliker et al., 1980; Deák and Beuchat, 1993; Worobo and Splittstoesser, 2005; Thomas and Davenport 1985; James and Stratford, 2003; Martorellet al., 2007). Products with low pH values, high sugar or salt content, added with weak acids as preservatives are: fruit juices, filling creams, sauces, salad dressings, sugar syrups, molasses,

honey, malt extract, jams, jellies, packaged products, dried fruit, carbonated soft drinks and ketchup (James and Stratford, 2003). *Z. rouxii* and *Z. bailii* are the most tolerant to high sugar concentrations being able to develop also in matrices containing 60-70% of sugars (Martorell et al., 2007). Moreover, *Z. bailii* is able to tolerate the presence of preservatives at concentrations in some cases well beyond the legally permitted thresholds (Steels et al., 1999). The bases of this extreme resistance to chemical preservatives and especially to weak acids are to be attributed to the presence of a small percentage of strains, within this species, with a low intracellular pH value (Stratford et al., 2013). These low values give to these strains the ability to reduce the accumulation of weak acids in the cytoplasm, thus determining resistance to acids but not to other inhibitors such as ethanol, aldehydes, esters, ethers, ketones (Stratford et al., 2013). Moreover, *Z. bailii* exhibits a high tolerance to SO_2, and to low-water activity (a_w) foods (James and Stratford, 2003).

Spoilage activity by these yeasts can lead to off flavors, defects and excessive gas production in foods (Martorell et al., 2007). The amount of CO_2 generated can be sufficient to cause distortion of packaging, rupture of cans or of kegs and explosion of bottles (Grimbaum et al., 2004). This can lead to great economic losses for the company if the yeast spoilage leads to the recall of the product. Moreover, the recall of a product is always related to the loss of image or brand for the company.

Based on these observations, in the last years alternative preservatives are being sought over traditional ones to reduce yeast spoilage events. EOs and EO compounds have shown to have an antifungal activity against *Z. bailii*, therefore EOs could be used as natural preservatives against yeast spoilage in foods and beverages rich in sugars (Ciani et al., 2010).

Object of the work

A study has been performed to evaluate the antifungal activity of seven EOs against 21 yeast spoilage isolates belonging to the species *Z. bailii* previously collected from contaminated filling creams for bakery products (Osimani et al., 2016) by using a disc diffusion assay. This test can provide useful information about the sensitivity of spoilage isolates of *Z. bailii* against different EOs, that could be used for the formulation of products with potential efficacy in the control of spoilage by *Z. bailii*.

Material and methods

Essential oils

Lime (*Citrus aurantifolia)*, Ginger (*Zingiber officinale*), Lemongrass (*Cymbopogon citratus*), Mandarin (*Citrus reticulata*), Cinnamon (*Cinnamonum zeylanicum*), Orange (*Citrus sinensis*) and Lemon (*Citrus limon*) were obtained from Sigma Aldrich (Saint Louis, USA) and their quality parameters (appearance, color, purity, odor, density, refraction index) were described in an accompanying technical report.

Yeast isolates

The 21 *Z. bailii* isolates have been collected from seven different batches of filling creams for bakery products as described by Osimani et al. (2017). The cultures were grown overnight at 25°C on YPD agar medium (yeast extract 10 g L^{-1}, peptone 20 g L^{-1}, D-glucose 20 g L^{-1}, agar 18 g L^{-1}) and the biomass obtained was diluted in sterile peptone water (peptone 1 g L^{-1}). The yeast suspensions have been adjusted at a final concentration of 10^7 cfu/ml adjusted according to the turbidity of 0.5 McFarland scale tube (Souza et al., 2007).

Disc diffusion assay

The disc diffusion assay was employed for the determination of antifungal activities of the EOs. Briefly, 100 µl of each suspension of the tested isolate previously adjusted to 1 x 10^7 cfu/ml was spread on YPD agar medium plate. Sterile filter paper discs of 6 mm of diameter (Schleicher & Schuell, Germany) were placed on the surface of the inoculated plates and soaked with 10 µl of the

oil. The plates were incubated at 25°C for 48 h. The diameters of the inhibition zones were measured in millimeters. All tests were performed in duplicate.

Statistical analysis

Statistical analysis one-way ANOVA was carried out using JMP, version 11.0.0 (SAS Institute Inc., Cary, NC, USA) to underline significant differences on the effect of different EOs against yeast isolates. The differences were considered significant for $P < 0.05$.

Results and Discussion

The results of the effectiveness of the antifungal activity of the EOs of lime, ginger, lemongrass, mandarin, cinnamon, orange and lemon are shown are shown in Table 1.

Isolate	Essential oil						
	Lime (mm)	**Ginger (mm)**	**Lemongrass (mm)**	**Mandarin (mm)**	**Cinnamon (mm)**	**Orange (mm)**	**Lemon (mm)**
z1	14±0.0 **cd**	0±0.0 **e**	25±1.4 **b**	0±0.0 **e**	48±0.7 **a**	20±0.0 **bc**	9±1.4 **d**
z2	14±0.0 **c**	0±0.0 **e**	22±0.0 **b**	0±0.0 **e**	45±1.4 **a**	17±1.4 **c**	9±1.4 **d**
z3	14±2.8 **bc**	0±0.0 **d**	21±1.4 **b**	0±0.0 **d**	46±2.8 **a**	16±2.8 **b**	8±0.0 **c**
z4	13±4.2 **cd**	0±0.0 **e**	26±2.8 **b**	0±0.0 **e**	43±1.4 **a**	20±2.8 **bc**	8±0.0 **de**
z5	15±1.4 **cd**	0±0.0 **e**	23±4.2 **b**	0±0.0 **e**	43±1.4 **a**	19±1.4 **bc**	9±1.4 **d**
z6	14±2.8 **bcd**	0±0.0 **d**	25±4.2 **b**	0±0.0 **d**	45±7.1 **a**	17±4.2 **bc**	8±0.0 **cd**
z7	17±1.4 **c**	0±0.0 **e**	24±2.8 **b**	0±0.0 **e**	45±2.8 **a**	20±0.0 **bc**	8±0.0 **d**
z8	14±2.8 **cd**	0±0.0 **e**	22±0.0 **b**	0±0.0 **e**	50±2.8 **a**	19±1.4 **bc**	10±0.0 **d**
z9	12±2.8 **cd**	0±0.0 **e**	26±2.8 **b**	0±0.0 **e**	48±2.8 **a**	19±1.4 **bc**	8±0.0 **d**
z10	14±2.8 **cd**	0±0.0 **e**	24±0.0 **b**	0±0.0 **e**	46±0.0 **a**	20±2.8 **bc**	10±2.8 **d**
z11	15±4.2 **cd**	0±0.0 **e**	24±2.8 **b**	0±0.0 **e**	45±1.4 **a**	21±1.4 **bc**	8±0.0 **de**
z12	14±2.8 **c**	0±0.0 **e**	23±1.4 **b**	0±0.0 **e**	53±1.4 **a**	21±1.4 **b**	8±0.0 **d**
z13	14±2.8 **bc**	0±0.0 **d**	20±2.8 **b**	0±0.0 **d**	46±2.8 **a**	22±2.8 **b**	9±1.4 **c**
z14	15±2.8 **bc**	0±0.0 **d**	22±2.8 **b**	0±0.0 **d**	49±7.1 **a**	21±1.4 **b**	8±0.0 **cd**
z15	16±0.0 **bc**	0±0.0 **d**	23±4.2 **b**	0±0.0 **d**	54±0.0 **a**	18±5.7 **bc**	8±0.0 **cd**
z16	15±1.4 **cd**	0±0.0 **e**	23±4.2 **b**	0±0.0 **e**	49±1.4 **a**	21±1.4 **bc**	8±0.0 **d**
z17	17±1.4 **b**	0±0.0 **d**	21±1.4 **b**	0±0.0 **d**	52±2.8 **a**	17±1.4 **b**	8±0.0 **c**
z18	14±2.8 **c**	0±0.0 **e**	21±1.4 **b**	0±0.0 **e**	49±1.4 **a**	17±1.4 **bc**	8±0.0 **d**
z19	13±1.4 **c**	0±0.0 **e**	21±1.4 **b**	0±0.0 **e**	49±1.4 **a**	21±1.4 **b**	8±0.0 **d**
z20	15±1.4 **c**	0±0.0 **e**	22±0.0 **b**	0±0.0 **e**	51±1.4 **a**	16±2.8 **c**	8±0.0 **d**
z21	17±1.4 **bc**	0±0.0 **e**	24±0.0 **b**	0±0.0 **e**	55±4.2 **a**	16±2.8 **c**	8±0.0 **d**

Table 1. Results of antifungal activity (inhibition zone) of EOs tested on 21 isolates of *Zygosaccharomyces bailii* collected from filling creams. For each isolate the averages of the inhibition zone (expressed in millimetres) due to the effectiveness of the EOs are submitted to statistical analysis of one way ANOVA. Significantly differences were considered for $P < 0.05$.

For each EO the average value and standard deviation of two replicas of the diameter measurements (in millimetres) of the inhibition zone are shown.

The statistical analysis one-way ANOVA of the data has shown a significant greater efficacy of cinnamon EO on the tested isolates (with a range of values of inhibition zone included between 43 and 55 mm) followed by lemongrass (20-26 mm) and orange (16-22 mm) EOs. Lime and lemon EOs have been shown to have a lower antifungal efficacy respect than cinnamon, lemongrass and orange

EOs; in fact, the values of their inhibition zone are between 20 and 26 mm and between 16 and 22 mm respectively.

Moreover, 100% of the tested isolates were not sensitive to the presence of ginger or mandarin EOs.

These results show the potential role of EOs (cinnamon, lemongrass, orange) to contrast *Z. bailii* spoilage, but the applicability of these EOs *in vivo* must be verified. Food matrices can alter the effectiveness of EOs, which may be lower than *in vitro* tests. Furthermore, the aromatic notes associate with EOs could limit the range of applications, through the modification of taste and flavour of the final product, which may not be liked by consumers.

Conclusions

Although different studies have shown that EOs have a good antimicrobial, only a few studies are available about the antifungal activity of EOs. Therefore, the use of EOs in food products against yeasts spoilage is still an uncommon practice. Moreover, there is a limited knowledge about the sensory impact of the use of EOs on foods. *Zygosaccharomyces* are important yeast spoilage but a few tests have been carried out to evaluate the sensitivity of strains of *Zygosaccharomyces* to different EOs. Mint, oregano and clove EOs are the only EOs tested against *Zygosaccharomyces*, and only mint EO was tested *in vivo* in apple juice.

The study that has been carried out has confirmed the antifungal activity of some EOs against different isolates of *Z. bailii*. However, although the results obtained on the antifungal activity of cinnamon, lemongrass and orange EOs against *Z. bailii* are encouraging, their impact and their applicability in food matrices remains to be verified *in vivo*, in which the pronounced aromatic notes associated with these two EOs could limit the possible range of applications.

Chapter 4.2 - The potential role of essential oils as preservatives against spoilage yeasts in yogurt

Introduction

Yogurt

Yogurt is a fermented milk that has been very popular for a long time in Mediterranean countries (the Balkans, North Africa), in central and southwest Asia (Mongolia, Turkey, Iraq, Iran, Syria) and in central Europe. In many of these countries, yogurt is still manufactured using traditional procedures. For yogurt production, milk with an appropriate fat content (typically < 0.5, 1, or 3.25%) and total solids content (typically 12 to 15% and adjusted by adding fat-free dry milk) is homogenized, pasteurized and cooled to 40 to 45°C before pumping into a vat. The milk is then inoculated with starter cultures namely *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. The inoculated milk is immediately pumped into a yogurt filler and then filled into cups, with or without fruit puree already present in the bottom of the cup, and the cups are then sealed. The filled and sealed cups are then transferred to a warm room for incubation (40-45°C). When the yogurt reaches the appropriate pH (typically about 4.6), the yogurt cups are transferred to refrigerated storage (Kosikowski and Mistry, 1997). Therefore, the modern yogurt production is a well-controlled process that utilizes milk, milk powder, sugar, fruit, flavour, colouring, emulsifiers, stabilizers, and specific cultures of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* as ingredients.

Although yeasts are considered contaminants in yogurt, non-lactose fermenting *Torulopsis* yeasts have been isolated from yogurt and it has been found that they helped the survival of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, possibly through the removal of some lactic acid (Soulides, 1955). However, the growth of yeasts in yogurt was found to limit the microbiological shelf life of

unflavored and strawberry yogurt during storage at 8°C or 20°C (Tirloni et al., 2015). Yeasts are found in raw and pasteurized milks at 10^1-10^3 cfu/ml (Fleet, 2011). However, their growth in milk during refrigerated storage is limited, as they are quickly overgrown by psychrotrophic bacteria. Nevertheless, milk is an excellent substrate for yeast growth, and in the absence of bacterial competition, they readily develop populations of 10^8-10^9 cfu/ml (Roostita and Fleet, 1996). However, thanks to their low pH, yogurt is an elective environment for the growth of yeasts because its acidity restricts bacterial competition.

The spoilage of yogurt by yeasts is generally recognized by the development of yeasty off-flavours, loss of texture quality due to gas production, and the swelling of the product container (Fleet, 2008; Ledenbach and Marshall, 2009). Yeast spoilage can arise from raw materials, such as fruits, and from ineffective cleaning and sanitation of processing equipment. When yogurt is produced by using good manufacturing practice, it should contain no more than 1 yeast cell for g and, if correctly stored under refrigeration (5°C), it may be expected a product shelf life from 3 to 4 weeks. Yeasts isolated from spoiled yogurts include *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, and various species of *Pichia* and *Candida* (Caggia et al., 2001; Vasdinyei and Deak, 2003).

Object of the work

A reason of the increasing of yeast spoilage events is related to the modern technologies in food production. Modern technologies in food manufacturing tend to utilize less severe processing conditions in order to preserve flavors, tastes, and natural colors of the products as much as possible. Moreover, new food formulations, such as the different types of yogurts added with juices and concentrated fruits, sugar syrups, and sliced fruits, have significantly contributed to the raise of spoilage events by yeasts. Yogurt is one of the most product subjected to yeast spoilage events, due to its intrinsically properties (such as low pH) that promote the growth of yeast compared to the bacteria.

In this context, the aim of this study was to assess the potential role of seven different EOs (cinnamon, ginger, lemongrass, mandarin, orange, lemon and lime) as preservatives in yogurt against spoilage yeasts. These EOs were chosen for their possible positive attribution to yogurt's flavor and odor. The antifungal activity of these EOs was evaluated by a disc diffusion assay on 74 spoilage yeast isolates collected from yogurts prepared with pasteurized buffalo milk. The tested yeasts belonged to the genera *Candida*, *Rhodotorula*, *Debaryomyces*, *Kluyveromyces* and *Yarrowia*. The best performing EOs were selected for further minimum inhibitory concentration (MIC) assay against a representative pool of the total yeast isolates, by using the micro-well dilution method. The potential role as preservatives of these selected EOs was tested also *in vivo*. The selected EOs were added in a lab-scale produced yogurt, intentionally contaminated by a selected yeast isolate, in order to confirm their effectiveness against yeast spoilage. A final acceptance test was carried out to evaluate the sensory impact of EOSs on the final product.

Material and methods

Essential Oils

The seven EOs used were described in chapter 4.1, paragraph “Essential oils”.

Yeasts isolates

Seventy-four yeast isolates have been previously collected from three different batches of spoiled yogurt (with yeasty off-flavours) produced with pasteurized buffalo milk by a local producer. The isolates were previously identified (data not yet published) and the species represented were *Candida sake* (4 isolates), *Candida parapsilosis* (2), *Candida intermedia* (1), *Candida pararugosa* (3), *Candida aaseri* (1), *Candida lusitaniae* (2), *Rhodotorula glutinis* (1), *Rhodotorula diobovata* (1), *Rhodotorula babjevae* (2), *Debaryomyces hansenii* (21), *Debaryomyces subglobosus* (1), *Kluyveromyces lactis* (1), *Yarrowia lipolytica* (28) and *Yarrowia deformans* (6).

The isolates were grown overnight at 25°C on YPD agar medium (yeast extract 10 g L^{-1}, peptone 20 g L^{-1}, D-glucose 20 g L^{-1}, agar 18 g L^{-1}). The biomass obtained was diluted in sterile peptone water (peptone 1 g L^{-1}) to obtain a final concentration of 10^7 cfu/ml adjusted adjusted according to the turbidity of 0.5 McFarland scale tube (Souza et al.,2007).

Disc diffusion assay

The disc diffusion assay was described in chapter 4.1, paragraph “Disc diffusion assay”. All isolates were tested for the seven EOs, and all tests were performed in duplicate. The best performing EOs have been selected for further analyses.

Determination of minimum inhibitory concentration (MIC)

According to Souza et al. (2005), a microplate bioassay (microdilution) was used to define the minimum inhibitory concentration (MIC) of lemongrass and cinnamon EOs against 20 selected yeast isolates. The EOs of cinnamon and lemongrass were assayed at concentrations of 10, 5, 2.5, 1.25, 0.62 and 0.31 µl/ml and the solutions have been prepared according to Souza et al. (2005). The microtiters were prepared by dispensing into each well 100 µl of Sabouraud broth (peptone 10 g L^{-1}, D-glucose 20 g L^{-1}) inoculated with the yeast inoculum prior to the assay. An aliquot (100 µl) of the EO solutions at each concentration was transferred into six consecutive wells to obtain the following final concentrations: 10, 5, 2.5, 1.25, 0.62 and 0.31 µl/ml. The final volume in each well was 200 µl. The solution having the highest concentration was added into the first well (final concentration 10 µl/ml), so that the smallest concentration was added into the sixth well (final concentration 0.31 µl/ml). A well, containing 200 µl of the sterile Sabouraud broth was also used as a negative control. Moreover, a well, containing 200 µl of Sabouraud broth inoculated with the yeast inoculum, was used as the positive control. The microplate was aseptically sealed, followed by mixing on a plate shaker (300 rpm) for 30 s, and incubated at 25°C for 48 h (Sahin et al., 2004; Viljoen et al., 2003). The MIC was defined as the lowest concentration of the EO able to provide visible yeast growth inhibition after the end of the incubation time (Cellini et al., 1996). All the tests were performed in duplicate.

Lab-scale yogurt production

The potential role of the selected EOs as preservatives in yogurt was tested directly in yogurts intentionally contaminated by a selected pool of yeast spoilage isolates. Yogurts were produced in a lab-scale by using pasteurized whole bovine (U.H.T.) milk (12.14% dry matter; 3.74% fat; 3.35% protein; 4.65% lactose; and 0.74% ash) and commercial starter cultures of *L. delbrueckii* subsp.

bulgaricus and *S. thermophilus* for yogurt production. In this trial three different spoilage yeast isolates belonging to the species *Debaryomyces hansenii* (isolate code 35), *Candida pararugosa* (isolate code 59) and *Yarrowia deformans* (isolate code 86) have been tested. A lab-scale yogurt production has been carried out adding 1.25 µl/ml of one EO (cinnamon or lemongrass) at milk (1 L), cultures starter strains (both at initial concentration of 10^6 cfu/ml) and one yeast spoilage isolate. Two different concentrations (10^2 and 10^4 cfu/ml) of inoculum was used for each isolate. In each trial each EO has been added at the beginning of the fermentation process. Moreover, a yogurt production has been performed without EO and yeast strains as a control. Another control was set up by adding EO to milk without yeast isolates. Moreover, yogurt inoculated with a mixture of the three selected yeast isolates at different concentrations (10^2 or 10^4 cfu/ml) and without EOs was produced. All samples have been performed in duplicate (34 total samples). All the samples were incubated at 45°C until to reach a pH value of 4.6. When the samples reached this pH value, they were stored at 4°C for 28 day. Chemical analysis (pH) and viable counts were performed at the beginning of the fermentation and after 8 h, 4 days, 8 days and 28 days from its start.

Enumeration of culturable bacteria and yeasts

During the fermentation and storage, 2 ml from all experimental yogurts were removed and analyzed for the enumeration of viable counts of yeasts (1 ml) and bacteria (1 ml) respectively. The 1 mL aliquots were serially diluted. Serial decimal dilutions were prepared in sterile 0.1% peptone solution and 0.1 mL of each serial dilution was inoculated by surface spreading onto specific solid media in duplicate. Bacteria were counted on M17 agar medium (Merck, Darmstadt, Germany) supplemented with 400 mg/L of cycloheximide to inhibit yeast growth, at 45°C under anaerobic conditions (Camu et al., 2008) for 48 h. Yeasts were counted on YPD agar medium (yeast extract 10 g L^{-1}, peptone 20

g L^{-1}, D-glucose 20 g L^{-1}, agar 18 g L^{-1}) supplemented with 200 mg/L of chloramphenicol to inhibit bacteria growth at 25 °C aerobically for 4 days. All samples were performed in duplicate.

pH measurement of yogurt

The pH of yogurt was measured at room temperature using a model 300 pH meter equipped with an HI2031 solid electrode (HI2031, Hanna Instruments, Padua, Italy). To this end, about 5 ml from all samples were removed. All tests were performed in duplicate.

Statistical analysis

Analysis of variance (ANOVA) was carried out using JMP, version 11.0.0 (SAS Institute Inc., Cary, NC, USA) to underline significant differences on the effect of different EOs against yeast isolates by using the disc diffusion assay. The differences were considered significant for $P < 0.05$.

Acceptance test

An acceptance test was used to evaluate the taste of the final product. To this end, the samples were served randomly to 10 untrained testers (5 male and 5 female). The testers were between 26 and 50 years old and have evaluated the sample by rating a vote from 1 to 9 for global liking for each one.

Results and Discussion

A disc diffusion assay has been carried out to test the antifungal activity of the 7 EOs against the 74 yeast isolates. The results obtained, expressed in millimetres of growth inhibition zone, were subsequently submitted to a statistical analysis of one way ANOVA (Supplementary Table 1). The statistical analysis of data has shown a significant greater antifungal activity of lemongrass and cinnamon EOs against the tested isolates. Furthermore, this statistical analysis has shown that lemongrass and cinnamon EOs were less effective against *Y. lipolytica* and *D. hansenii* isolates respectively. Lemongrass EO has exhibited a range of values of inhibition zone comprised from 22 to 71 mm, while cinnamon EO has shown a range of values comprised between 26 and 55 mm. Orange has been shown a quite good antifungal activity (range of values comprised between 4 and 30 mm) even if it was less effective against *D. hansenii* isolates. Lime, lemon, mandarin and ginger EOs have exhibited a low effectiveness against all the isolates tested. In fact, their inhibition zone values were the following: lime (0-17 mm); lemon (0-12 mm); mandarin (0-9 mm); ginger (0-8 mm).

Based on these results lemongrass and cinnamon EOs have been selected to determine their minimum inhibitory concentration (MIC) against a selection of 20 yeast isolates belonging to 14 different species isolated from the spoiled yogurt (Table 1). The isolates were selected to obtain a representative pool of the yeast species isolated. The selection was made as follows: one isolate was selected for the species that had few isolates out of 74 yeasts total, while two or three isolates were selected for more abundant species. One isolate was selected among the species *C. parapsilosis*, *C. sake*, *C. intermedia*, *C. pararugosa*, *C. aaseri*, *R. babjevae*, *R. diobovata*, *R. diobovata*, *K. lactis*, *D. subglobosus*. Two isolates were selected among the species *C. lusitaniae* (isolate codes 24 and 42) and *Y. deformans* (63, 86), while three isolates were selected among the species *D. hansenii* (35, 64, 65) and *Y. lipolytica* (46, 76, 89). All these isolates were selected on the bases of their growth inhibition zone values in the presence of lemongrass (from 29 to 71 mm) or cinnamon (from 29 to 50

mm) EOs and of their statistical analysis. The selection was made in order to obtain a pool of isolates with different sensitivity to EOs, although the choice of less sensitive EO isolates was privileged.

Isolate code	**Species**
19	*Candida parapsilosis*
24	*Candida lusitaniae*
33	*Candida sake*
35	*Debaryomyces hansenii*
38	*Candida intermedia*
42	*Candida lusitaniae*
46	*Yarrowia lipolytica*
51	*Rhodotorula babjevae*
59	*Candida pararugosa*
61	*Kluyveromyces lactis*
63	*Yarowia deformans*
64	*Debaryomyces hansenii*
65	*Debaryomyces hansenii*
72	*Rhodotorula glutinis*
73	*Rhodotorula diobovata*
74	*Candida aaseri*
76	*Yarrowia lipolytica*
86	*Yarrowia deformans*
89	*Yarrowia lipolytica*
93	*Debaryomyces subglobosus*

Table 1. List of the selected yeast isolates to be submitted to a microplate bioassay

In addition to the disc diffusion assay, the minimum inhibitory concentration (MIC) of lemongrass and cinnamon EOs against the 20 selected yeast isolates was evaluated. MIC was obtained by using a micro-well dilution method [as described in paragraph “Determination of minimum inhibitory concentration (MIC)”]. The results of these trials are shown in the following table (Table 2).

Isolate code	Species	Lemongrass EO		Cinnamon EO	
		MIC	MIC	MIC	MIC
19	*Candida parapsilosis*	0.62	0.62	1.25	1.25
24	*Candida lusitaniae*	1.25	1.25	1.25	1.25
33	*Candida sake*	1.25	1.25	0.31	0.31
35	*Debaryomyces hansenii*	1.25	1.25	1.25	1.25
38	*Candida intermedia*	1.25	1.25	0.31	0.31
42	*Candida lusitaniae*	1.25	1.25	1.25	1.25
46	*Yarrowia lipolytica*	0.62	0.62	0.62	0.62
51	*Rhodotorula babjevae*	0.62	0.62	0.62	0.62
59	*Candida pararugosa*	1.25	1.25	1.25	1.25
61	*Kluyveromyces lactis*	1.25	1.25	1.25	1.25
63	*Yarowia deformans*	0.62	0.62	0.62	0.62
64	*Debaryomyces hansenii*	1.25	1.25	0.62	0.62
65	*Debaryomyces hansenii*	1.25	1.25	0.62	0.62
72	*Rhodotorula glutinis*	0.31	0.31	0.31	0.31
73	*Rhodotorula diobovata*	0.31	0.31	0.31	0.31
74	*Candida aaseri*	0.62	0.62	0.62	0.62
76	*Yarrowia lipolytica*	1.25	1.25	0.62	0.62
86	*Yarrowia deformans*	1.25	1.25	1.25	1.25
89	*Yarrowia lipolytica*	0.62	0.62	0.62	0.62
93	*Debaryomyces subglobosus*	1.25	1.25	0.62	0.62

Table 2. Lemongrass and Cinnamon EO MIC on yeast isolates determined by microplate assay. Concentration of EOs are expressed in µl/ml.

The two EOs have shown similar results; Thirteen isolates had the same MIC for both EOs. Considering their antifungal activity demonstrated by the disc diffusion assay, these results are not unexpected. Both EOs were able to inhibit the yeast growth for all yeast isolates tested at the concentrations varying from 0.31 to 1.25 µl/ml. The lowest concentration tested (0.31 µl/ml) of

lemongrass EO has inhibited the growth of two yeast isolates, both belonging to the genus *Rhodotorula* (isolate codes 72 and 73). Cinnamon EO at the lowest concentration tested is effective against four yeast spoilage: isolate codes 33 and 38 (genus *Candida*), and isolate codes 72 and 73 (genus *Rhodotorula*). A concentration less than or equal to 0.62 µl/ml of lemongrass or cinnamon EO has an effective antifungal activity against eight and thirteen isolates respectively. The growth of six yeast isolates was inhibited at 1.25 µl/ml for both EOs. Therefore, in this test, cinnamon EO confirmed to have the best antifungal activity compared to the other EOs. The isolate code 33 (*Candida sake*) was the strain that showed the main difference between the MIC values of the two EOs. The isolate was more sensitive to cinnamon EO (0.31 µl/ml MIC value) than lemongrass EO (1.25 µl/ml MIC value). The growth of four isolates belonging to the genus *Debaryomyces* has been inhibited up to 0.62 µl/ml of lemongrass EO, proving to be a less sensitive genus to this EO compared to other tested ones.

Based on these results, the next step was to test the effectiveness of lemongrass and cinnamon EOs at 1.25 µl/ml as preservatives against three selected yeast spoilage isolates *in vivo*. The selected yeast isolates belonged to the species *D. hansenii* (isolate code 35), *C. pararugosa* (isolate code 59) and *Y. deformans* (isolate code 86). This selection was carried out to obtain a representative pool of yeast isolates, on the base of MIC values of both EOs, which prevents visible growth. The isolates selected have shown an inhibition growth at 1.25 µl/ml for both EOs. One of them, isolate code 35, was the only one belonging to the species *D. hansenii*, a species widely represented on the 74 starting isolates. Isolates 86 and 59 were selected to represent the genus *Yarrowia* and *Candida* respectively, two genus to which many of the 74 starting isolates belong.

A lab-scale yogurt production was carried out as described in paragraph “Lab-scale yogurt production”. Yogurt samples were intentionally contaminated with one of three selected yeasts isolate.

Figures 1 and 2 shows the pH trend of all samples at the beginning of fermentation and after 8 hours, 4, 8 and 28 days.

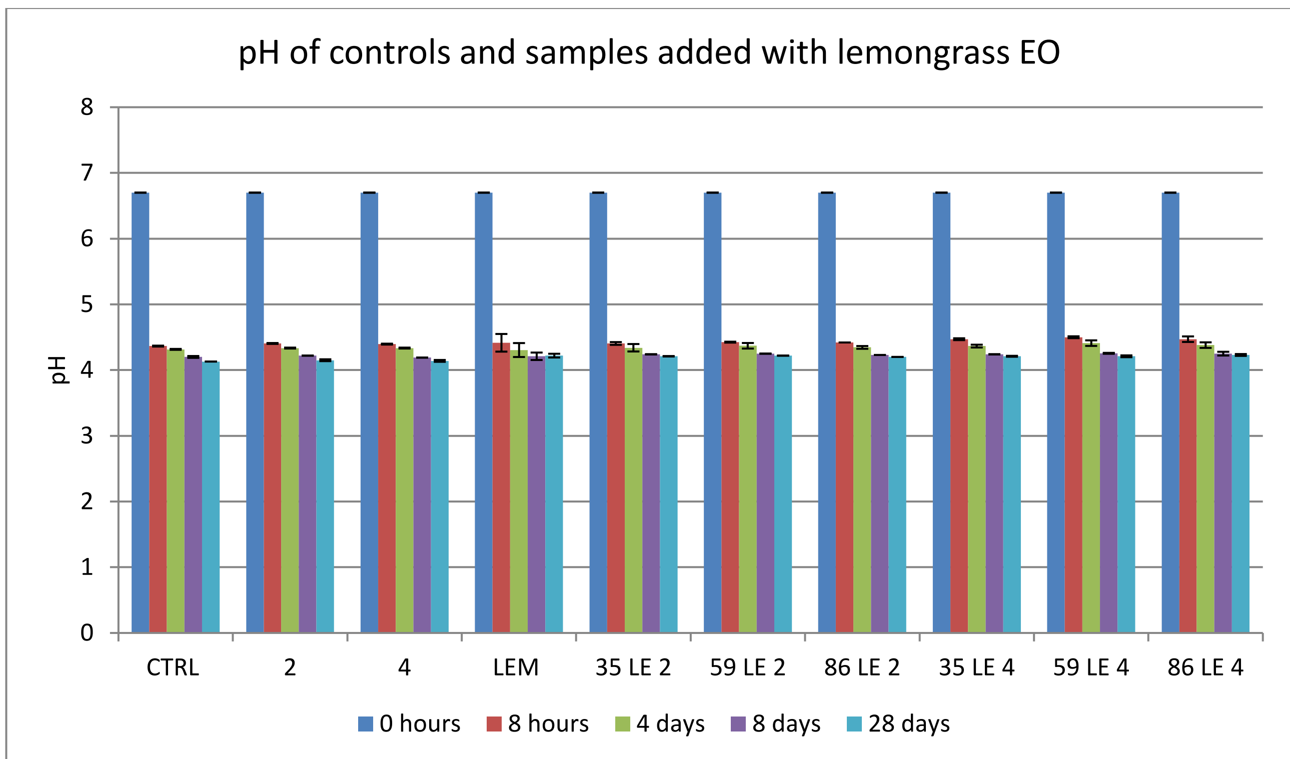

Figure 1. pH trend of controls and samples added with lemongrass EO at the beginning of the fermentation and after 8 hours, 4, 8 and 28 days. (CTRL): negative control (yogurt); (2) and (4): positive controls (yogurt with addition of 10^2 or 10^4 cfu/ml respectively of a mixture of the three selected yeast isolates); (LEM): negative control of lemongrass EO (yogurt uncontaminated with the addition of lemongrass EO); (35 LE 2): *D. hansenii*, isolate code 35, inoculated at 10^2 in yogurt added with lemongrass EO (1.25 µl/ml) ; (35 LE 4): *D. hansenii*, isolate code 35, inoculated at 10^4 cfu/ml in yogurt added with lemongrass EO (1.25 µl/ml); (59 LE 2): *C. pararugosa*, isolate code 59, inoculated at 10^2 in yogurt added with lemongrass EO (1.25 µl/ml); (59 LE 4): *C. pararugosa*, isolate code 59, inoculated at 10^4 cfu/ml in yogurt added with lemongrass EO (1.25 µl/ml); (86 LE 2): *Y. deformans*, isolate code 86, inoculated at 10^2 in yogurt added with lemongrass EO (1.25 µl/ml); (86 LE 4): *Y. deformans*, isolate code 86, inoculated at 10^4 cfu/ml in yogurt added with lemongrass EO (1.25 µl/ml).

From Figure 1 emerges that all the samples had the same trend. At the beginning of the fermentation pH values were 6.7; after 8 hours of incubation at 45°C, pH of all samples reached values lower than 4.6. Therefore, considering that the fermentation was over, these samples has been stored at 4°C until the 28th day. During the storage at 4°C, the pH values continued to decrease slowly, reaching measures of about 4.2 for all samples.

Figure 2 shows the pH trend of controls and samples added with cinnamon EO.

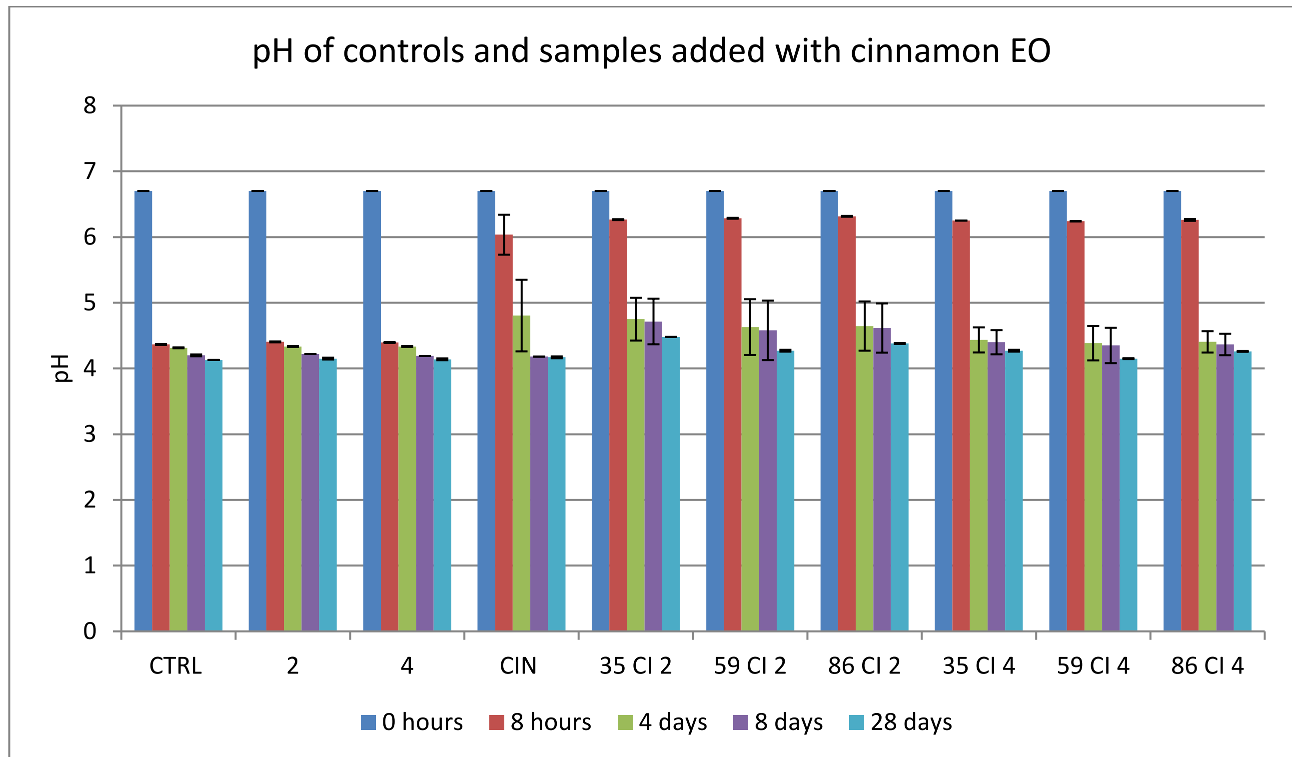

Figure 2. pH trend of controls and samples added with cinnamon EO at the beginning of the fermentation and after 8 hours, 4, 8 and 28 days. (CIN): negative control of cinnamon EO (yogurt uncontaminated with the addition of cinnamon EO); (2) and (4): positive controls (yogurt with addition of 10^2 or 10^4 cfu/ml respectively of a mixture of the three selected yeast isolates); (35 CI 2): *D. hansenii*, isolate code 35, inoculated at 10^2 in yogurt added with cinnamon EO (1.25 µl/ml); (35 CI 4): *D. hansenii*, isolate code 35, inoculated at 10^4 cfu/ml in yogurt added with cinnamon EO (1.25 µl/ml); (59 CI 2): *C. pararugosa*, isolate code 59, inoculated at 10^2 in yogurt added with cinnamon EO (1.25 µl/ml); (59 CI 4): *C. pararugosa*, isolate code 59, inoculated at 10^4 cfu/ml in yogurt added with cinnamon EO (1.25 µl/ml); (86 CI 2): *Y. deformans*, isolate code 86, inoculated at 10^2 in yogurt added with cinnamon EO (1.25 µl/ml); (86 CI 4): *Y. deformans*, isolate code86, inoculated at 10^4 cfu/ml in yogurt added with cinnamon EO (1.25 µl/ml).

It's clear that these samples had a very different pH trend from the samples shown in Figure 1. The initial pH value of 6.7 decreased very slowly during fermentation. After 8 hours of incubation at 45°C, all the samples had pH values higher than 6.0, demonstrating an incomplete fermentation. Therefore, these samples were not stored at 4°C but were incubated again at 45°C. The pH analyses were performed after 4 days because, during the incubation, the samples showed a texture close to the milk's one, demonstrating an incomplete fermentation once again. It has been hypothesized that

this event could be related to a high antimicrobial activity of cinnamon EO. After 4 days, most of the samples had reached a pH value lower than 4.6. These samples were stored at 4°C for another 24 days (28 days in total since the beginning of the fermentation process). The other ones (CIN and 35 CI 2) were incubated until their pH values reached 4.6 or less. These sample were stored at 4°C after 8 days.

During fermentation, the concentration of yeasts and bacteria was monitored by the enumeration of their viable counts. These analyses were carried out at the beginning of the fermentation and after 8 hours, 4, 8 and 28 days.

As to be expected the cinnamon EO has shown a high antimicrobial activity that inhibits the growth of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* starter strains and thus preventing a correct fermentation of the milk and of yogurt production. The concentration of bacteria in all yogurt samples with the addition of cinnamon EO was between 10 or 10^2 cfu/ml (data not shown) during the whole monitoring time. These results have confirmed the data obtained by Tzortzakis and Moritz on the antimicrobial activity of cinnamon EO in yogurt (Tzortzakis, 2009; Moritz et al., 2015). At the same time, cinnamon EO has been shown to confirm its antifungal activity *in vivo*, because each yeast strain was not detected during the whole fermentation time (data not shown).

Lemongrass EO has been shown not to inhibit the growth of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* starter strains. Indeed, the concentrations of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were stable for all the samples, added with lemongrass EO, during the fermentation process and subsequent storage at 4°C, remaining between 10^7 (inoculum) and 10^9 cfu/ml (after 8 hours) (data not shown). Regarding the antifungal activity of lemongrass EO, the concentrations of the different yeast isolates were related to the species. In fact, in the samples contaminated with the isolate of *D. hansenii* (isolate 35) or *Y. deformans* (isolate 86), the yeast cells were not detected during the whole monitoring time, confirming the effectiveness of the lemongrass EO as antifungal at 1.25 µl/ml against these species *in vivo* (data not shown). In the samples inoculated with the *C. pararugosa*

isolate (isolate 59), conversely, yeast cells were detected in all analyses carried out. The concentrations of *C. pararugosa* in yogurt samples intentionally contaminated by it (inoculum of 10^2 or 10^4 cfu/ml) after 4, 8 and 28 days from the beginning of the fermentation process are shown in the Figure 3.

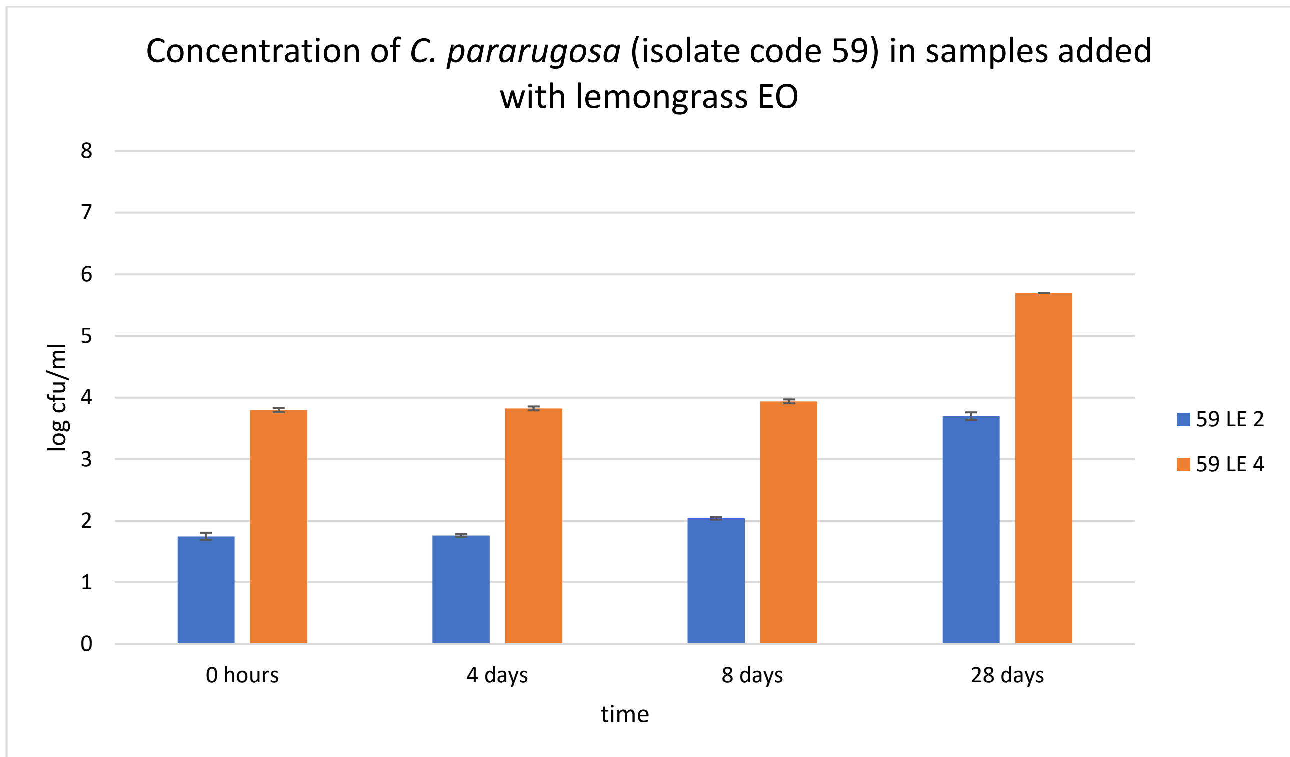

Figure 3. Evaluation of the concentration of *C. pararugosa* (isolate code 59) after 4, 8 and 28 days from the beginning of milk fermentation in intentionally contaminated yogurt samples added with lemongrass EO. (59 LE 2): *C. pararugosa*, inoculated at 10^2 cfu/ml in yogurt samples added with lemongrass EO (1.25 µl/ml); (59 LE 4): *C. pararugosa*, inoculated at 10^4 cfu/ml in yogurt samples added with lemongrass EO (1.25 µl/ml)

The results have demonstrated that lemongrass EO slows the growth of these yeast cells. After 8 days at 4°C the yeast concentrations in the two samples only slightly increased. After 28 days stored at 4°C the increasing is clear. The 59 LE 2 sample went from a value of 10^2 cfu/ml to almost 10^4 cfu/ml, while the 59 LE 4 sample has reached a value close to 10^6 cfu/ml, from a value of 10^4 cfu/ml. Overall, these results confirm the antifungal activity of lemongrass EO, performed by inhibiting or slowing the growth of yeast spoilage in yogurt. However, the effectiveness of this antifungal activity is species-dependent. In fact, lemongrass EO has shown the efficacy of its antifungal activity *in vivo*

against *D. hansenii* and *Y. deformans* spoilage isolate but not against *C. pararugosa* spoilage isolate. This aspect could limit the use of this EO as a preservative in food and beverages as it may not guarantee a complete inhibition of yeast spoilage growth.

In order to evaluate the sensory impact of lemongrass and cinnamon EOs on the final product, an acceptance test was performed (as described in paragraph "Acceptance test"). 10 untrained testers rated all the samples by voting each of them for global liking. Acceptance test results showed that all the samples added with an EO (lemongrass or cinnamon) were rated negatively (average score 4). On the contrary, the negative and positive controls were evaluated positively (average score above 7). Therefore, despite the antifungal activity of lemongrass and cinnamon EO at a concentration of 1.25 µl/ml was confirmed *in vivo* in yogurt samples, its presence in the final product seems to lead to a worsening of its taste.

Conclusions

Disc diffusion assay has been carried out to evaluate the antifungal activity of seven different EOs (lemon, lime, mandarin, ginger, orange, lemongrass and cinnamon) *in vitro* against 74 different yeast spoilage isolates belonging to different species. The results obtained from these trials have demonstrate that the antifungal activities of lemongrass and cinnamon EOs are the most effective against these yeast spoilage isolates. These conclusions are achieved based on the values of inhibition zone obtained with this disc diffusion assay.

Moreover, lemongrass and cinnamon EOs have been tested to assess the minimum inhibitory concentration (MIC) against a selection of 20 yeast isolates from the 74 total yeast spoilage isolates. Both EOs, were able to inhibit the yeast growth for all yeast isolates tested with the minimum concentration of 1.25 µl/ml.

Based on these results, a laboratory-scale yogurt production was carried out to evaluate the antifungal activity of lemongrass and cinnamon EOs *in vivo*. Although the results have been encouraging, their applicability seems to be limited. In fact, cinnamon EO has been shown to have antimicrobial activity against *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* starter strains, leading to an incomplete milk fermentation. Therefore, cinnamon EO is not suitable as preservative in yogurt, but considering its antifungal activity it could be used as a preservative against yeast spoilage in other foods or beverages. By contrast, lemongrass EO did not influence milk fermentation and yogurt production, its antifungal activity has been confirmed *in vivo* against the species *D. hansenii* and *Y. deformans*, but not for the species *C. pararugosa.*

Overall, these results demonstrated the possible application of lemongrass EO as a preservative in yogurt, although its effectiveness is species-dependent. The final sensory test, which aimed to determine the global-liking of the yogurt produced on a laboratory scale, showed that the presence of EO (lemongrass or cinnamon) determines a worsening of the taste of the final product. Therefore,

lemongrass EO at this concentration (1.25 µl/ml) could be used to preserve the microbial stability of the yogurt but its pronounced aromatic notes limit its application. Considering the negative impact of lemongrass EO on the global pleasantness of the final product, and its antifungal activity against different species of yeast spoilage, further research could be carried out to expand the application of lemongrass EO in the production of other foods and beverages.

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Chapter 5 - *Brettanomyces* spoilage in Albanian wines assessed by culture-dependent and culture-independent methods

Object of the work

This chapter, containing a study already published on Journal of Food Science, is about the evaluation of *Brettanomyces* spoilage in Albanian wines by combining culture-dependent and culture-independent methods.

In the Albanian winemaking industry, awareness of the potential detrimental effects of *Brettanomyces* activity in wines is very low. No studies have been conducted to evaluate the extension of this phenomenon in Albanian wines, especially in those produced from autochthonous cultivars, which have a higher economic value. Considering the increasing spread of *Brettanomyces* spoilage in wine industry all over the world, it is important to enhance the knowledge about the extension of wine contamination by these yeasts. Although a large number of culture-dependent techniques are available to assess the presence of this undesired yeast during the winemaking processes, in several case *Brettanomyces* is undetectable; The corresponding final products are affected by phenolic odours caused by the presence of these yeast species (Laforgue and Lonvaud-Funel, 2012; Serpaggi et al., 2012). This phenomenon has been explained by the capacity of *Brettanomyces* to enter in the "viable-but-not culturable" state (VBNC), in which the cells cannot divide on a specific media for *Dekkera*/*Brettanomyces bruxellensis*, even if they are still alive and maintain the metabolic activities and cell functions (Agnolucci et al., 2010; Divol and Lonvaud-Funel, 2005; Du Toit et al., 2005). Overall, the detection of *Brettanomyces* is difficult and it is important to enhance the knowledge of measures and tools to evaluate its presence. This work has aimed to assess the extension of spoilage by *Brettanomyces* in Albanian wines using a combined approach of culture-dependent (viable plate counting) and culture-independent methods (qPCR).

Paper*: Brettanomyces* Spoilage in Albanian Wines Assessed by Culture-Dependent and Culture-Independent Methods

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Practical Application: The awareness of *Brettanomyces* spoilage in the Albanian winemaking industry is very low. This study represents the first contribution to understand the extent of this spoilage yeast in Albanian autochthonous cultivars, which tend to have high economic value, to ensure product quality and safety. qPCR is confirmed to be a very sensitive method to rapidly detect *Brettanomyces* spoilage in wine samples.

Abstract

In the Albanian winemaking industry, there is little awareness of the potential detrimental effect of *Brettanomyces* in wines. The aim of this study was to detect and quantify *Brettanomyces* cells in 22 Albanian bottled wines, representing all the viticultural areas of Albania. A combined approach, including culture-dependent (viable plate counting) and culture-independent (qPCR) methods, was applied. Spoilage indicators (ethylphenols and total and volatile acidity), as well as the primary factors known to influence the growth of *Brettanomyces* in wine (pH, SO2, and ethanol concentration), were also investigated. *Brettanomyces* was detected in only five (one Merlot, four Sheshi i Zi) out of 22 samples analyzed using viable counting, with loads ranging from 1.30 ± 0.03 log CFU/mL to 3.99 ± 0.00 log CFU/mL, whereas it was never detected in the Kallmet samples. When qPCR was applied, *Brettanomyces* cells were detected and quantified in all of the samples with a generally low load ranging from 0.47 ± 0.13 to 3.99 ± 0.01 log cells/mL. As a general trend, the loads of spoilage by this yeast were low (1.92 log cells/mL), with the exception of five samples that were also positive by plate counting. A positive correlation between the growth of this spoilage yeast on *Dekkera*/*Brettanomyces* differential media and its detection at high levels by qPCR was observed. A significant positive correlation between *Brettanomyces* and the concentration of ethylphenols and volatile acidity was also found. In summary, the results of this study demonstrated the low incidence of *Brettanomyces* spoilage yeasts in Albanian red wines.

Keywords: Albanian wine, *Brettanomyces*, culture-dependent method, qPCR, 4-ethylphenol

Introduction

The increasing demand for quality wines by consumers has decreased the tolerance to aspects that formerly were not considered to be defects. Most of these are due to yeast activity, such as the slight haziness in bottled wines or the release of volatile phenols in tainted wine. Although *Dekkera/Brettanomyces* spp. have been known since the beginning of the 20th century (Henschke, Curtin, and Grbin, 2007; Krumbholz and Tauschanoff, 1933), they have only attracted the attention of wine technologists during the last decades. *Dekkera/Brettanomyces* spp. are currently regarded to be the most likely large microbiological problems of modern oenology, causing large economic losses in the wine sector worldwide (Boulton, Singleton, Bisson, and Kunkee, 1995; Fugelsang, 1997). As expected, within this group, *Brettanomyces bruxellensis* has been associated with various forms of wine spoilage, including cloudiness and a range of off-flavors (Malfeito-Ferreira, 2011).

For cultural or ethnic reasons, there is little difference between what is perceived as spoilage or beneficial activity (Fleet, 1992). For instance, the production of 4-ethylphenol (4-EP) by *Dekkera/Brettanomyces* spp. in red wines is only considered to be spoilage when this secondary metabolite occurs at levels higher than 620 μg/L (Chatonnet, Boidron, and Dubourdieu, 1993; Chatonnet, Dubourdie, Boidron, and Pons, 1992). Above this threshold, wines are clearly substandard for some consumers, due to "horsesweat" off-odors that are detrimental to the aroma profile (especially in red wines) (Chatonnet, Dubourdieu, and Boidron, 1995; Suárez, Suárez-Lepe, Morata, and Calderón, 2007). However, at less than 400 μg/L, this yeast metabolite contributes favorably to the complexity of wine aroma by imparting aromatic notes of spices, leather, smoke, or game, which are appreciated by most consumers.

In addition to its production of 4-EP, *B. bruxellensis* has the ability to spoil wine through a number of additional activities. Among these, the production of volatile acids has a negative impact on the fermentative performance of yeast, which when excessive, is a spoilage trait. Both bacteria and yeasts

can form acetic acid at any time of the winemaking process, from the very first stage (in grapes) until the end product (bottled wine). Species of *Brettanomyces* are strongly acidogenic, producing large amounts of acetic acid (Ciani and Ferraro, 1997; Fugelsang, Osborn, and Muller 1993). The production of acetate is thought to be a consequence of the blockage of the acetaldehyde oxidative pathway, caused by an insufficient activity of the enzyme acetyl-CoA synthetase that converts acetate to acetyl-CoA (Conde et al., 2007; Gerós, Azevedo, and Cássio, 2000). A two- to four-fold increase in the volatile acidity and acetic acid concentration of spoiled wine after 30 to 60 days incubation at 25 °C was observed by Chandra, Barata, Ferreira-Dias, Malfeito-Ferreira, and Loureiro (2014). Acetic acid production under high glucose concentrations has previously been reported from other studies (Gerós, Cássio, and Leao, 2000; Suárez et al., 2007).

Sulfur dioxide is the primary preservative used in wines, and *B*. *bruxellensis* has been regarded to be either resistant or sensitive (Loureiro and Malfeito-Ferreira, 2006). Some authors report its sensitivity to free sulfite at concentrations higher than 30 mg/L (Chatonnet et al., 1992; Gerbaux, Vincent, and Bertrand, 2002), thus explaining the frequent isolation of this yeast from sulfur dioxide unprotected wines (Heresztyn, 1986). Recent reports clearly recognize the sensitivity or resistance to SO_2 as a strain specific character of the *Brettanomyces* yeasts (Conterno, Joseph, Arvik, Henick-Kling, and Bisson, 2006; Vigentini, Joseph, Picozzi, Foschino, and Bisson, 2013). Sulfur dioxide proved to have a significant ($P < 0.05$) negative linear and quadratic effect on yeast growth and 4-EP production, respectively. In particular, it has been demonstrated that at pH 3.50, the concentrations of sulfur dioxide higher than 20 mg/L induce an immediate loss of yeast cell cultivability under growth at permissive levels of ethanol (Chandra et al., 2014). Increasing the levels of ethanol also has shown an inhibitory effect on *Brettanomyces*. Chandra et al. (2014) found that when the percentages are as high as 15% (v/v), low viable counts of the cells, close to the detection limit, were seen, as well as a lack of production of measurable amounts of 4-EP (Chandra et al., 2014). A problem in detecting a reliable load of *Brettanomyces* is their capacity to enter a physiological state of very low metabolic

activity and no growth on culture media, referred to as “viable-but-not culturable” (VBNC) (Millet and Lonvaud-Funel, 2000). This indicates that the classical methods used to enumerate and isolate these microorganisms might not be able to detect cells in this state. Sulfur dioxide has been shown to induce the VBNC state of the wine spoilage yeast *Brettanomyces* (Agnolucci et al., 2010; Du Toit, Pretorius, and Lonvaud-Funel, 2005; Serpaggi et al., 2012). Serpaggi et al. (2012) described the ability of several strains of *B. bruxellensis* to enter the VBNC state after a sulfite treatment ranging from 0.3 to 1.0 mg/L of molecular SO_2. Therefore, the quantitative polymerase chain reaction (qPCR) is undoubtedly a valid alternative to rapidly detect and quantify the *Brettanomyces* cells, including those in the VBNC state (Willenburg and Divol, 2012). In the Albanian winemaking industry, awareness of the potential detrimental effects of *Brettanomyces* activity in wines is very low, and, to our knowledge, no studies have been conducted to evaluate the extension of this phenomenon in Albanian wines, especially those produced from autochthonous cultivars, which tend to have a higher economic value. Given this premise, in this study, 22 Albanian bottled wines, produced in different areas of the country with different winery practices, were collected and analyzed for the presence of *B. bruxellensis* cells using a combined approach including culture-dependent (viable plate counting) and culture-independent (qPCR) methods. Key factors known to influence the growth of *Brettanomyces* yeast in wine, such as pH, SO_2, and ethanol concentration as well as spoilage indicators, including total and volatile acidity, and ethylphenols (EPs) were also analyzed.

Materials and methods

Wine Samples

Twenty-two samples of monovarietal wines from the native Albanian varieties “Merlot” (four samples), “Kallmet” (six samples), and “Sheshi i Zi” (12 samples) were collected. They were chosen from different wine grape-growing regions in Albania (Kullaj et al., 2012) and included various

vintages from 2013 to 2016 (Table 1). In addition, the producers were asked to fill out a questionnaire related to the percentage of grapes infected by *Botrytis cinerea* based on their approximate evaluation, and the results are reported in Table 1.

No.	Code	Viticultural Area	Variety	Vintage	% *Botrytis cinereal* infection
1	M1	Sarande	Merlot	2016	5
2	M2	Durres	Merlot	2016	n.r.
3	M2	Tirane	Merlot	2015	0.50
4	M4	Tirane	Merlot	2016	0.50
5	K1	Lezhe	Kallmet	2015	n.r.
6	K2	Lezhe	Kallmet	2015	n.r.
7	K3	Koplik	Kallmet	2015	n.r.
8	K4	Koplik	Kallmet	2016	n.r.
9	K5	Shkoder	Kallmet	2014	n.r.
10	K6	Shkoder	Kallmet	2015	n.r.
11	SH1	Tirane	Sheshi i Zi	2015	10
12	SH2	Tirane	Sheshi i Zi	2016	40
13	SH3	Tirane	Sheshi i Zi	2015	n.r.
14	SH4	Tirane	Sheshi i Zi	2015	n.r.
15	SH5	Sarande	Sheshi i Zi	2016	5
16	SH6	Tirane	Sheshi i Zi	2015	0.50

17	SH7	Tirane	Sheshi i Zi	2016	0.50
18	SH8	Tirane	Sheshi i Zi	2016	60
19	SH9	Tirane	Sheshi i Zi	2016	50
20	SH10	Tirane	Sheshi i Zi	2013	n.r.
21	SH11	Shijak	Sheshi i Zi	2016	n.r.
22	SH12	Vlore	Sheshi i Zi	2014	n.r.

Tab. 1 Albanian wine samples analyzed. n.r. not detected

Analytical methods

The chemical analysis of wines was performed using classical methods based on The Compendium of International Methods of Analysis of Wines and Musts, OIV (International Organisation of vine and wine, 2011). Respectively, the pH was measured using a pH-meter from Metrohm (Herisau, Switzerland) as described by OIV-MA-AS313-15, and the total acidity was based on OIV-MA-AS313-01:R2015. The total and free SO_2 were measured as described by OIV-MA-AS323-04B:R2009, whereas the alcohol content and volatile acidity were determined as described by OIV-MA-AS312-01A (OIV Method A2/1978 – Resolution 377/2009) and OIV-MA-AS313-02: R2015, respectively. The molecular SO_2 was determined from the free SO_2 and the pH using the Henderson–Hasselbalch equation (Boulton, Singleton, Bisson, and Kunkee, 1999).

Plate counting of *Brettanomyces* spp. in wines

The cultivable fraction of the *B. bruxellensis* population potentially occurring in the 22 wine samples was enumerated by the plate count method on *Dekkera/Brettanomyces* differential media (DBDM) (Rodrigues, Gonçalves, Pereira-da-Silva, Malfeito- Ferreira, and Loureiro, 2001) as described by Tofalo, Schirone, Corsetti, and Suzzi (2012). Ten-fold serial dilutions of the wine samples were prepared in sterile peptone water (peptone 1 g/L); 100 μL of each undiluted wine sample and serial dilutions were plated in duplicate and incubated at 25 °C for 10 days.

DNA Extraction

Ten milliliters of each wine sample were centrifuged at 13,000 rpm for 5 min to collect the cells. The supernatant was discarded, and the DNA was extracted from the cell pellet using a Commercial Kit (E.Z.N.A. soil DNA Kit; Omega Bio-tek, Norcross, GA, U.S.A.) according to the manufacturer's instructions. The quantity and purity of the resulting DNA extracts were determined as described by Sambrook et al. (1989) using a Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, U.S.A.).

Construction of qPCR standard curve

To create a standard curve for the absolute quantification of *B. bruxellensis* cells in wine samples, filter sterilized "Montepulciano" red wine was artificially contaminated with the strain *B. bruxellensis* D46 maintained at the yeast collection of the Dept. of Life and Environmental Sciences (DiSVA), Polytechnic Univ. of Marche and subcultured on DBDM. The amount of inoculated *B. bruxellensis* D46 cells per milliliters of wine was determined using the Thoma cell counting chamber and checked

for viability by traditional microbiological viable cell enumeration on DBDM. DNA extraction from 10 mL of artificially contaminated wine, as well as the assessment of DNA purity and concentration, were performed as previously described. The qPCR standard curve was created by plotting the Ct values of the qPCR performed on 10-fold serial dilutions of the extracted DNA against the number of cells per reaction. The amplification efficiency (E) was estimated from the slope of the standard curve using the formula $E = 10^{(-1/\text{slope})} - 1$. To determine the detection limit of qPCR, the standard curve was created covering the range from 1 to 10^6 cells of *B. bruxellensis* D46 per reaction.

qPCR quantification

The absolute quantification of *B. bruxellensis* cells in the wine samples was performed using aMastercyclerR _ ep realplex machine (Eppendorf, Hamburg, Germany). The amplification reactions were performed in a total 10 μL reaction volume including 5 μL of qPCR Green Master Mix LRox, 2X (Biotechrabbit GmbH, Hennigsdorf, Germany), 900 nM of forward primer (DBRUXF), and 300 nM of reverse primer (DBRUXR) (Phister and Mills, 2003) following the thermal cycling program described by Tofalo et al. (2012). Four microliters of each wine DNA extract and tenfold serial dilutions of the DNA extract from *B. bruxellensis* D46 cells, covering a range of 1 to 106 cells per reaction, were run in parallel. The specificity of the amplification reaction was checked by melting temperature analysis obtained with the temperature gradually increasing from 60 °C to 95 °C by 0.5 °C/s. The MastercyclerR _ ep Realplex software performed the baseline and threshold calculation automatically. The quantity of the *Brettanomyces* cells per milliliters of wine sample was calculated on the basis of the standard curve slope obtained as described above. All the samples, including the blank control (qPCR mixture added with molecular grade water instead of DNA extract), were processed in triplicate, and the results were expressed as the mean value ± standard deviation

Determination of 4-EP and 4-EG

The 4-EP and 4-ethylguaiacol (4-EG), that are volatile compounds, were extracted using a headspace-solid-phase microextraction technique and analyzed by gas chromatography. The fiber used in this study has the following characteristics: divinylbenzene/ carboxen/polydimethylsiloxane (DVB/CAR/PDMS), 50 to 30 μm, StableFlex/SS, 1 cm (Supelco, Bellefonte, PA, U.S.A.). The holder used was for manual injection and was also supplied by Supelco.

Five milliliters of each sample were placed into a 10-mL vial containing 2.5 g of NaCl and a magnetic stirrer, and the vial was hermetically sealed with a PTFE-faced silicone septum. Before the extraction step, the samples were equilibrated for 10 min at 25 °C and magnetically stirred at 200 rpm. The DVB/CAR/PDMS fiber was inserted through the vial septum and exposed to the sample headspace for 40 min at 55 °C. For quantification, aliquots of the wine samples were spiked before their extraction with the 3-octanol used as an internal standard (1.6 mg/L). The analyses were conducted on a GC-2014 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector, a glass Supelcowax-10 column (60 m × 0.32 mm × 0.25 mm) from Supelco. Nitrogen was used as the carrier gas (flow rate of 3.74 mL/min). The fiber was inserted on the GC-port (230 °C) for 5 min to transfer the analytes to the chromatographic column. A split/splitless injector was used in the splitless mode: 60 seconds splitless; injection and detector temperature, 230 °C. The column program was: 50 °C for 1 min, and increased 2 °C/min to 200 °C and maintained at 200 °C for 20 min. The compounds were identified and quantified by comparison with external calibration curve of known compounds (4-EP and 4-EG).

Statistical analysis

Linear regression was used to perform the statistical analysis using the MS Excel 2013 Data Analysis ToolPak.

Results and discussion

Detection of *Brettanomyces* by plate counting and qPCR

A combination of culture-dependent (plate counting) and culture-independent (qPCR) methods was used to detect and quantify *B. bruxellensis* in 22 wine samples covering all the viticultural areas of Albania (Merlot, Kallmet, and Sheshi i Zi). To date, this spoilage yeast has been detected and isolated in almost every wine-producing area of the world (A´ vila and Ayub, 2013; Fugelsang, 1997). However, to our knowledge, there is a lack of information regarding the spoilage of Albanian wines due to the *Brettanomyces* yeast. Given this premise, this study represents a first attempt to understand the extent at which this yeast occurs in Albanian wines, especially those produced with autochthonous cultivars in a way to establish a strategy for adequate control measures. The results of the occurrence of *Brettanomyces* yeast are reported in Table 2.

Wine sample codes	**Viable counts (log CFU/mL)**	**qPCR counts (log cells/mL)**
M1	**<1**	**1.92±0.01**
M2	**<1**	**0.47±0.13**
M3	**3.43±0.01**	**3.02±0.02**

M4	**<1**	**1.04±0.13**
K1	**<1**	**1.13±0.10**
K2	**<1**	**1.18±0.06**
K3	**<1**	**1.44±0.10**
K4	**<1**	**1.50±0.11**
K5	**<1**	**1.17±0.06**
K6	**<1**	**1.20±0.13**
SH1	**<1**	**0.93±0.06**
SH2	**<1**	**1.13±0.05**
SH3	**<1**	**1.87±0.03**
SH4	**<1**	**1.19±0.06**
SH5	**<1**	**0.90±0.07**
SH6	**1.30±0.03**	**2.47±0.05**
SH7	**2.20±0.01**	**2.46±0.02**
SH8	**2.97±0.01**	**2.98±0.02**
SH9	**<1**	**1.45±0.08**
SH10	**3.99±0.00**	**3.99±0.01**
SH11	**<1**	**1.90±0.02**
SH12	**<1**	**1.18±0.14**

Tab.2 Comparison of cell counts of *Brettanomyces* spp. assessed by viable plate method on DBDM and qPCR. Data are means ± SD.

Overall, *Brettanomyces* yeasts were detected in only 22% (5 of 22) of the analyzed samples regardless of the grape variety. If these results are compared to those of the studies available, Albanian red wines are characterized by an extent of *Brettanomyces* spoilage slightly lower than that of Brazilian (A′ vila and Ayub, 2013) and French red wines (Gerbaux, Jeudy, and Monamy, 2000), respectively. Wines from different grape varieties were analyzed. In this study *Brettanomyces* was detected in only one Merlot (M3) and four Sheshi i Zi (SH6, SH7, SH8, SH10) wine samples by traditional plate counting, with loads ranging from 1.30 ± 0.03 log CFU/mL to 3.99 ± 0.00 log CFU/mL in samples SH6 and SH10, respectively. None of the Kallmet samples (K1–K6) were positive for the growth of *Brettanomyces* on the differential medium used.

Contamination by *Brettanomyces* could potentially occur at any stage of the winemaking process, starting from the soil, leaves and grapes and ending with the winery equipment, barrels, and bottling rooms (Oelofse, Pretorius, and Toit, 2008). *Brettanomyces* is rarely detected on grapes, and, to our knowledge, this spoilage yeast has only been isolated in a sole study (Renouf and Lonvaud- Funel, 2007) after an enrichment step. This result could explain the dominance of the highly abundant species over the minority species, such as *B. bruxellensis*, which may remain undetected on grapes. As reported by Chatonnet et al. (1992), the load of *B. bruxellensis* cells increase when the growth conditions are more favorable. These include the completed alcoholic fermentation, a low level of residual sugars, and the availability of the nutrients from the lysed cells of competing yeast.

In addition, wooden barrels used to age the red wines are recognized as the most critical factor for *Brettanomyces* contamination due to their distinctive porous microstructure, which is highly permeable to small amounts of oxygen, thus encouraging the growth of this facultative anaerobe yeast. In addition, the porous microstructure of the wooden barrels could be responsible for an incomplete removal of spoilage yeasts during the sanification processes (Agnolucci et al., 2017; Oelofse et al., 2008; Pollnitz, Pardon, and Sefton, 2000). In this study, only two Merlot samples (M3, M4) from the same cellar (vintages 2015 and 2016) were aged in barrels, but surprisingly, only sample

M3 was characterized by a high *Brettanomyces* load (3.43 ± 0.01 log CFU/mL). Taillandier (2007) suggested a possible positive correlation between *Brettanomyces* spoilage and *Botrytis cinerea* infection in grapes. On the basis of the responses of the producers to the questionnaire (Table 1), 10 wine samples were produced with a low portion of grapes affected by *Botrytis* (0.5–10%), with the exception for the samples SH2, SH8, and SH9, produced with grapes largely affected by this plant pathogen (40%, 60%, and 50%, respectively). Among these latter group, only sample SH8, which was produced from the highest proportion of damaged grapes (60%), reacted positively for *Brettanomyces* with a load approximately 2.97 ± 0.01 log CFU/mL. Unfortunately, the data concerning *B. cinerea* infection were not reported for the sample SH10, which resulted in the highest load of *Brettanomyces* (3.99 ± 0.00 log CFU/mL). However, in addition to use of wooden barrels and *B. cinerea* infection of grapes, a number of other factors, such as grape variety, geographical location, temperature, winery environment, and equipment, may have an influence on wine spoilage by *Brettanomyces*. Interestingly, three (M3, SH6, SH7) out of the five wines positive for this spoilage yeast were produced in the same cellar, thus suggesting a potential role of the winery environment or equipment of such a cellar. However, the fact that no Kallmet wines collected from different cellars and vintages were positive for the occurrence of *Brettanomyces* suggested that even the grape variety might have had an influence on the occurrence and distribution of this spoilage yeast in the Albanian red wines analyzed, with Kallmet being a less susceptible grape variety to spoilage in respect to the other two.

Despite being effective, the plate counting method is time consuming and, in the case for *Brettanomyces*, it can lead to underestimations, due to the potential of this yeast to enter into a VBNC state. This latter condition is typically induced by sulfur dioxide addition (Agnolucci et al., 2010; Divol and Lonvaud-Funel, 2005; Du Toit et al., 2005; Serpaggi et al., 2012). However, as previously demonstrated by numerous authors (Du Toit et al., 2005; Li,Mendis, Trigui, Oliver, and Faucher, 2014; Serpaggi et al., 2012), the VBNC state is reversible, and the cells can easily return to an actively

metabolizing and culturable state once the stress factors are removed. To prevent wine spoilage and the subsequent economic losses, there is a need for a fast and accurate method to detect and quantify *Brettanomyces* cells in wine. To date, a number of culture-independent methods, such as PCR, nested PCR and PCR-denaturing gradient gel electrophoresis (PCR-DGGE)(Campolongo, Rantsiou, Giordano, Gerbi, and Cocolin, 2010; Cocolin, Rantsiou, Iacumin, Zironi, and Comi, 2004; Ibeas, Lozano, Perdigones, and Jimenez, 1996; Martorell et al., 2006; Renouf et al., 2006) have been used to overcome the difficulties described above. However, these methods are often characterized by a low detection limit ($\geq 10^4$ CFU/mL) of the spoilage yeasts (Loureiro and Malfeito-Ferreira, 2006). Because the critical load of *Brettanomyces* for wine spoilage is approximately 10^3 cells/mL of wine (Grbin and Henschke, 2000), culture-independent methods with detection limits bellow this threshold are needed. qPCR undoubtedly represents an extremely valid alternative, because it detect the organism when as low as 1 to 10 cells/mL are present in the wine, as previously reported by numerous authors (Phister and Mills, 2003; Tofalo et al., 2012; Vendrame, Manzano, Comi, Bertrand, and Iacumin, 2014; Willenburg and Divol, 2012).

In this study, the standard curve, generated by plotting the Ct values of the qPCR performed on 10-fold serial dilutions of the DNAextracted directly from artificially contaminated wine against the number of cells per reaction, showed a linear correlation over a range of seven orders of magnitude, from 1 to 10^6 cells per reaction (Figure S1). Following the procedure described by Tofalo et al. (2012), the standard curve was created by inoculating *Brettanomyces* cells directly in red wine instead of water to avoid possible differences in the amplification efficiency of the Albanian wine samples and standard dilutions due to red wine PCR inhibitors, such as polyphenols, polysaccharides or tannins. In addition, a good correspondence was observed in the number of *B. bruxellensis* cells artificially inoculated into red wine assessed by cell counting with a Thoma chamber (6.99 ± 0.23 log cells/mL) and the plate counting method (6.97 ± 0.12 log CFU/mL), thus suggesting that the standard curve was created by the inoculation of almost exclusively living cells. The melting temperature analysis

showed that all the PCR products (wine samples and standards) had a melting temperature of 80.7 ± 0.4 °C. The efficiency of the qPCR reaction was 0.98 with the slope and R2 equal to −3.37 and 1.000, respectively, whereas its detection limit, defined as the lowest number of detectable *Brettanomyces* cells per reaction, was 1. This latter result is consistent with those of previous studies using the same primer set and qPCR procedure to detect and quantify *B. bruxellensis* in wine, where a limit of detection as low as 1 (Phister and Mills, 2003) and 10 (Tofalo et al., 2012) cells per reaction were observed.

The results from qPCR differed from those obtained by viable counting. This revealed the occurrence of *Brettanomyces* cells in all the samples analyzed (Table 2), with log number of cells per milliliter ranging from 0.47 ± 0.13 in sample M2 to 3.99 ± 0.01 in sample SH10. As a general trend, the loads of this spoilage yeast were low (≤ 1.92 log cells/mL), except for five samples that were also positive using plate counting. In more detail, in four of these samples, the loads of *Brettanomyces* cells assessed by viable counting and qPCR overlapped, whereas in sample SH6, the number of cells detected by qPCR was markedly higher (2.47 ± 0.05 log cells/mL) than that resulting from viable counting (1.30 ± 0.03 log CFU/mL). This latter result, together with the qPCR detection of *Brettanomyces* in 17 samples that were found to be negative by viable counting, could be due to the amplification of DNA from the VBNC, dormant and even dead but still intact cells (Agnolucci, Scarano, Rea, Toffanin, and Nuti, 2007; Campolongo et al., 2010; Vendrame et al., 2014), thus leading to a potential overestimation of metabolically active cells. To overcome this problem, some authors have recently suggested the combined use of propidium monoazide (PMA) treatment with qPCR as a solution to successfully enumerate the *B. bruxellensis* viable cells in wine and beer (Vendrame et al., 2014).

Correlation between the occurrence of *Brettanomyces* yeasts and the concentration of ethylphenols in wine

B. bruxellensis is recognized as the primary wine spoilage yeast able to convert hydroxycinnamic acids into volatile phenols, such as 4-EP and 4-EG. When perceived in red wines, these latter compounds are described as having a "Brett" taint, consisting of a characteristic set of off-flavors and aromas, for example, "animal," "barnyard," or "horse sweat" (Chatonnet et al., 1992, 1995; Oelofse et al., 2009). Brett taint or Brett character is responsible for substantial economic losses to the wine industry (Capozzi et al., 2016).

In Table 3, the amount of 4-EP and 4-EG detected in the 22 Albanian wine samples assessed by SPME-GC is reported. In more detail, 4-EP was detected in all the Merlot (M1–M4), two Kallmet (K5, K6), and 10 Sheshi i Zi (SH1, SH2, SH3, SH5, SH6, SH7, SH8, SH10, SH11, SH12) wine samples at levels ranging from 14 (sample SH3) to 641 μg/L (sample SH10). The average concentration of this compound per sample was 139 μg/L, whereas that per grape variety was 217, 23, and 171 μg/L for Merlot, Kallmet, and Sheshi i Zi, respectively. When compared to the previous compound, 4-EG occurred less frequently in the Albanian wines analyzed, because it was detected in three Merlot (M1, M3, M4), one Kallmet (K1), and three Sheshi i Zi samples (SH2, SH3, SH9), at levels ranging from 23 (sample M4) to 499 μg/L (sample M3). The average concentration per sample was 38 μg/L, whereas that per grape variety was 138 μg/L for Merlot, 20 μg/L for Sheshi i Zi, and 7 μg/L for Kallmet. When the amount of ethylphenols found in the Albanian red wines is compared to that of wines from other countries, notable differences emerge, with those from Albania showing substantially lower levels of these phenolic compounds. In contrast, in red wines from France, an average content of 440 μg/L was reported (Chatonnet et al., 1992), whereas those from Austria (Cabernet Sauvignon and Cabernet Sauvignon-Merlot), Brazil, and Australia, were characterized by an average content of 490 (Henschke et al., 2004), 593 (Ávila and Ayub, 2013), and 795 μg/L (Pollnitz et al., 2000), respectively. The same studies also found the mean concentration values for 4-EG to be

65 and 99 μg/L, respectively, for Brazilian and Australian red wines. In addition, Albanian wines were characterized by a considerably lower average concentration of this additional spoilage indicator. As reported by a few authors, the sensory preference threshold of 4-EP and 4-EG is 620 and 140 μg/L, respectively (Chatonnet et al., 1995; Suárez et al., 2007). As a whole, Albanian wines had ethylphenols concentrations below both of these threshold limits, except sample SH10 (Sheshi i Zi, vintage 2013), which was characterized by a 4-EP concentration (641 μg/L) slightly over the threshold limit, and sample M3 (Merlot, vintage 2015) with a concentration of 4-EG 3.6-fold (499 μg/L) higher than the preference threshold. As expected, both these samples were characterized with the highest load of *Brettanomyces* spp. cells (above 3 log CFU/mL). Overall, a significant positive correlation was seen between the occurrence of *Brettanomyces* cells and the concentration of 4-EP (R = 0.71, P < 0.001). It has previously been reported that in wine, *Brettanomyces* can produce characteristic off-flavors if the loads of at least 3 log cells/mL are reached (Chatonnet et al., 1995; Fugelsang and Zoecklein, 2003; Grbin and Henschke, 2000), thus confirming the results from this study. However, as shown in Table 2 and 3, ethylphenols were also detected in some samples that were characterized with very low loads of *Brettanomyces* cells (e.g., samples M2, SH1, SH5, and SH12). This result could be attributed to either nonspoilage yeasts, such as *Candida* and *Pichia* (Ávila and Ayub, 2013; Chatonnet et al., 1992; Dias, Pereira-da-Silva, Tavares, Malfeito-Ferreira, and Loureiro, 2003) or even lactic acid bacteria (Couto et al., 2006; De Las Rivas, Rodríguez, Curiel, Landete, and Munoz, 2008).

In addition to microbiological factors, even the grape variety could have an influence on the production of ethylphenols. In previous studies that examined different wine-producing regions all over the world, it was suggested that the concentration of ethylphenols varied significantly among grape varieties. Kheir, Salameh, Strehaiano, Brandam, and Lteif (2013) reported that the availability of phenolic precursors (phenolic acids) significantly influences the production of ethylphenols. As early as 1998, Goldberg and colleagues reported a very high concentration of these precursors in

Cabernet Sauvignon and Merlot grape varieties (Goldberg, Ng, Karumanchiri, Diamandis, and Soleas, 1996). Consistent with the later study, a high mean concentration of ethylphenols was observed in the Merlot samples analyzed even in this study.

Wine sample codes	4-Ethylphenol (µg/L)	4-Ethylguaiacol (µg/L)
M1	209±15.7	29±11.3
M2	183±25.7	n.d.
M3	408±44.5	499±67.4
M4	70±17.3	23±2.7
Average Merlot samples	217±140.6	138±241.1
K1	n.d.	43±6.1
K2	n.d.	n.d.
K3	n.d.	n.d.
K4	n.d.	n.d.
K5	127±39.1	n.d.
K6	12±7.4	n.d.
Average Kallmet samples	23±51.0	7±17.6
SH1	357±0.4	n.d.
SH2	22±7.8	59±2.2
SH3	14±3.9	46±5.2
SH4	n.d.	n.d.

SH5	133±4.4	n.d.
SH6	301±43.9	n.d.
SH7	200±23	n.d.
SH8	88±16.3	n.d.
SH9	n.d.	137±21.8
SH10	641±34.2	n.d.
SH11	140±4.2	n.d.
SH12	162±38.4	n.d.
Average SH samples	171±179.8	20±42.1
Average M+K+SH samples	139±165.9	38±107.9

Table 3. The concentration of 4-ethylphenol and 4-ethylguaiacol detected in the 22 Albanian wine samples. n.d. not detected

Influence of chemical parameters on the prevalence of *Brettanomyces* in wine

Volatile acidity refers to a number of wine distillable acids, including acetic, lactic, formic, butyric, and propionic acids, both in the free state and combined as salts (Oelofse, Lonvaud-Funel, andDu Toit, 2009). Acetic acid is undoubtedly the primary component of the volatile acidity of grape musts and wines. It often occurs at levels ranging from 0.2 to 0.6 g/L in wine, but under certain conditions, such as in ice wines (Erasmus et al., 2004) and botrytized wines, higher concentrations up to 2.1 g/L can be reached (OIV 2010). For this reason, the International Organisation of Vine and Wine (OIV) established that for most wines, except for particularly elaborated old wines, the volatile acidity

should not exceed 1.2 g/L (expressed as acetic acid) (International Organisation of Vine and Wine, 2011). As shown in Table 4, the volatile acidity of the 22 Albanian wines varied from 0.807 (sample SH5) to 1.109 (sample K2) g/L. A significant positive correlation between the presence of *Brettanomyces* and the volatile acidity ($R = 0.67$, $P < 0.001$) was found, which was consistent with the literature reviewed.

An effective SO_2 management can keep *Brettanomyces* yeasts under control (Curtin, Varela, and Borneman, 2015). This indicates that the measurement of the ratio of free and total SO_2 can be successfully used to control this spoilage yeast. Indeed, in the presence of active cells, the amount of free SO_2 tends to drop. The free SO_2 of the 22 Albanian wines analyzed in this study (Table 4) ranged from 9.6 to 81.6 mg/L. In comparison with other studies that observed the sensitivity of *Brettanomyces* to free SO_2 higher than 30 mg/L (Chatonnet et al., 1992; Gerbaux et al., 2002), in this study, all the samples did not show a significant negative correlation between the load of *Brettanomyces* and the levels of free SO_2 in wines even though it is notable that the samples with a high load of *Brettanomyces* (M3, SH6, SH7, SH8, SH10) had free SO_2 levels lower than 20 mg/L consistent with the results of Chandra et al. (2014) where higher concentrations inhibited cell culturability. Recent reports show the sensitivity and resistance to SO_2 to be a strain-specific character (Conterno et al., 2006; Vigentini et al., 2013). The form of SO_2 is very important. Free SO_2 is referred as sum of major SO_2 species at the wine pH, bisulfite ($HSO3-$), and SO_2, which typically exists in its neutral molecular form (Waterhouse, Sacks, and Jeffery, 2016). The antimicrobial activity of SO_2 is primarily due to the molecular form, because this neutral species is able to readily diffuse across the cell membrane (Divol, Du Toit, and Duckitt, 2012). The molecular SO_2 in our samples ranged from 0.10 to 0.41 mg/L, with only two samples above this level, 1.10 mg/L (SH5) and 2.16 mg/L (M1), respectively. Different studies revealed that levels of SO_2 above 0.8 mg/L can affect the culturability of different *Brettanomyces* strains, so a VBNC state was induced, and at level up to 2.1 mg/L, a loss of both culturability and viability was observed (Agnolucci et al., 2010; Coulon, Raffestin, Bellan,

Lonvaud-Funel, and Inra, 2013). In our samples, as expected, no significant negative correlation was observed between the molecular SO_2 and the load of *Brettanomyces*, because the levels of molecular SO_2 are below 0.8 mg/L.

Brettanomyces species are not among the dominant yeasts during must fermentation, probably due to their low growth rate, which renders these microorganisms unable to compete with *Saccharomyces cerevisiae* (Ciani and Ferraro, 1997; Froudiere and Larue, 1988; Gerós, Cássio, et al., 2000). However, *Brettanomyces* are often found in wine, a stressful environment for most microorganisms, due to its low amounts of residual sugars and high levels of ethanol (Froudiere and Larue, 1988). More than a decade ago, Silva and colleagues (2004) demonstrated that in the absence of glucose, *B. bruxellensis* exhibited a higher ability to grow at high ethanol concentrations, because it is less susceptible to the toxic effects of ethanol. In addition, in media with low sugar supplies, such as wine, *Brettanomyces* spp. are able to express high-affinity monosaccharide uptake systems (Silva et al., 2004). These results are consistent with those of this study, which substantially confirmed the high tolerance of *Brettanomyces* to ethanol. As expected, alcohol content higher than 12% (v/v) was found in the samples with high loads of these spoilage yeasts, such as samples M3, SH6, SH7, SH8, and SH10.

Sample	pH	Total acidity (g/L)	Volatile acidity (g/L)	Alcohol content % (v/v)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Molecular SO_2 (mg/L)
M1	**3.30**	**5.98±0.11**	**0.809±0.0020**	**12.87±0.015**	**68.80±2.26**	**251.20±20.4**	**2.16±0.04**
M2	**3.73**	**4.95±0.00**	**0.971±0.0015**	**12.09±0.001**	**22.40±4.53**	**105.60±9.05**	**0.27±0.03**
M3	**3.67**	**6.10±0.11**	**0.916±0.0017**	**13.65±0.003**	**14.40±2.26**	**81.60±2.260**	**0.20±0.02**
M4	**3.84**	**5.25±0.13**	**0.993±0.0015**	**12.96±0.005**	**44.80±27.1**	**158.40±42.9**	**0.41±0.13**

K1	**4.11**	**4.40±0.11**	**1.099±0.0001**	**12.69±0.001**	**38.40±9.05**	**140.80±9.05**	**0.19±0.02**
K2	**4.11**	**4.05±0.20**	**1.109±0.0003**	**12.52±0.001**	**81.60±11.3**	**238.40±20.3**	**0.41±0.03**
K3	**3.80**	**5.05±0.04**	**0.988±0.0002**	**14.82±0.001**	**20.80±6.79**	**75.20±2.260**	**0.21±0.03**
K4	**4.01**	**4.93±0.04**	**1.055±0.0002**	**15.37±0.000**	**19.20±0.00**	**76.80±4.530**	**0.12±0.00**
K5	**3.95**	**5.25±0.13**	**1.026±0.0015**	**13.30±0.001**	**16.00±4.53**	**97.60±6.790**	**0.12±0.02**
K6	**4.00**	**5.58±0.04**	**1.032±0.0001**	**14.64±0.001**	**16.00±0.00**	**92.80±0.000**	**0.10±0.00**
SH1	**3.86**	**5.15±0.11**	**1.002±0.0020**	**14.29±0.000**	**17.60±6.79**	**76.80±22.60**	**0.16±0.03**
SH2	**3.90**	**4.53±0.04**	**1.034±0.0013**	**14.46±0.001**	**17.60±6.79**	**65.60±6.790**	**0.14±0.03**
SH3	**3.47**	**5.40±0.08**	**0.878±0.0016**	**13.93±0.014**	**9.60±0.000**	**160.00±4.53**	**0.21±0.00**
SH4	**3.46**	**5.05±0.04**	**0.886±0.0013**	**12.52±0.006**	**9.60±0.000**	**128.00±22.6**	**0.21±0.00**
SH5	**3.28**	**5.88±0.04**	**0.807±0.0014**	**12.61±0.001**	**33.60±15.8**	**241.60±2.26**	**1.10±0.26**
SH6	**3.61**	**5.30±0.16**	**0.922±0.0016**	**13.93±0.000**	**14.40±2.26**	**78.40±6.790**	**0.22±0.02**
SH7	**3.58**	**5.23±0.04**	**0.917±0.0017**	**12.69±0.001**	**17.60±2.26**	**89.60±4.530**	**0.29±0.02**
SH8	**3.44**	**6.88±0.04**	**0.825±0.0017**	**14.20±0.000**	**17.60±2.26**	**84.80±2.26**	**0.40±0.03**
SH9	**3.69**	**6.68±0.12**	**0.905±0.0018**	**14.64±0.001**	**22.40±0.00**	**89.60±0.000**	**0.29±0.00**
SH10	**3.61**	**8.20±0.04**	**0.836±0.0011**	**12.78±0.000**	**16.00±4.53**	**70.40±9.05**	**0.25±0.04**
SH11	**3.68**	**7.08±0.04**	**0.891±0.0013**	**12.52±0.002**	**24.00±11.3**	**86.40±9.05**	**0.32±0.08**
SH12	**3.74**	**6.15±0.00**	**0.938±0.0014**	**14.82±0.001**	**20.80±2.26**	**72.00±11.31**	**0.24±0.01**

Tab. 4 Chemical parameters of the 22 Albanian wine samples

Conclusions

This study represents the first effort to determine the frequency of the occurrence of *Brettanomyces* in Albanian wines, especially those produced using autochthonous cultivars. The detection and quantification of *Brettanomyces* was performed in 22 wine samples representing all the viticultural areas of Albania, such as Merlot, Kallmet, and Sheshi i Zi, through a combination of culture-dependent (plate counting) and culture-independent (qPCR) methods. qPCR detected *Brettanomyces* cells in all of the samples. Generally, the load of this spoilage yeast was found to be very low with the exception of five samples, which were also positive by plate counting. The Albanian red wines investigated were characterized by a substantially low concentration of ethylphenols when compared to wines from other countries. As expected, a significant positive correlation between the presence of *Brettanomyces* and the volatile acidity was found. In addition, the high tolerance of *Brettanomyces* to ethanol was also confirmed.

Finally, even if the overall data of this study indicated the low occurrence of *Brettanomyces* in the Albanian red wines analyzed, further studies aimed to identify the source of this microbial contamination are necessary to prevent eventual wine spoilage and substantial economic losses.

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Chapter 6: Conclusions

The work presented in this Ph.D. thesis aims to enhance the knowledge of yeasts in from two points of view: i) yeasts with a pro-technological role in food industry and ii) yeasts with a detrimental effect on food industry.

Regarding the pro-technological role, two different studies have been carried out.

In the first, different craft beers were tested to detect and quantify Nicotinamide Riboside (NR), a recently discovered form of vitamin B_3 related to several health benefits, and its precursors Nicotinamide Mononucleotide (NMN) and Nicotinamide Adenine Dinucleotide (NAD^+). This work has allowed to demonstrate that production and release of NAD^+ is species-dependent. Only in beers produced using different strains of *S. cerevisiae* this molecule was detected. It was possible to demonstrate that NR and NMN productions are *S. cerevisiae* strain-dependent. The data obtained from a laboratory-scale beer production show the effects of hop addition during beer fermentation on the *S. cerevisiae* metabolism. Hop addition seems to have a potential role enhancing the ability of *S. cerevisiae* to release NR in the final product and therefore to increase its NR amount. Moreover, hop addition seems at the same time to reduce the amount of NAD^+ in the final product. Furthermore, other fermentation trials performed in YPD medium suggested that the ability of NR releasing by *S. cerevisiae* is strain-independent and wort-independent too. Overall, the results obtained point out that further analyses are needed to understand the interference of hop with the *S. cerevisiae* yeast metabolism. The knowledge of this interaction could be the first step for the production of a beer with a high vitamin B_3 content and possibly with a low alcohol content.

Kefir is a well-known food for its beneficial effects related with its microbiota. A study on kefir grains microbiota and on the microbial dynamics occurring during the production of kefir through traditional and backslopping methods was performed. In particular, the core mycobiota of kefir grains was composed mainly by three different species: *Kazachstania unispora*, *Saccharomyces cerevisiae* and

Kluyveromyces marxianus. Backslopped kefir has shown to contain a very similar mycobiota to the kefir grains samples, while the relative abundance of yeast species in the traditional kefir is different. Besides, the yeast amount decreases from grains to the backslopped kefir. Furthermore, the kefir drinks obtained with the two different methods were different in terms of physico-chemical and nutritional features as well as for volatile profiles. The results confirm the usefulness of potential scale-up methodology of backslopping method. In fact, the data obtained from the physico-chemical and nutritional analyses have shown that backslopped kefir enhances nutritional value of the final drink for most of the parameters evaluated, compared to traditional kefir. Moreover, phenethyl alcohol and phenethylacetate have been detected in kefir drinks. These compounds are related to health benefits thanks to their antimicrobial activity on the consumers.

Considering the health benefits related to kefir and the data obtained in these studies, it is important to increase the knowledge about kefir drinks, in particular backslopped kefir.

EOs have known for their antimicrobial activity, while only a few studies have been carried out to evaluate their antifungal activity. Two different studies have been performed to test the effectiveness of seven different EOs (lime, lemon, lemongrass, cinnamon, ginger, orange and mandarin) against different isolates. In the first study, EOs were tested against twenty-one different isolates of *Zygosaccharomyces bailii* collected from filling creams. Lemongrass, cinnamon and orange have demonstrated to be more effective than the others EOs. In the second study the EOs were tested against seventy-four isolates collected from contaminated yogurt produced from buffalo milk. The results have demonstrated a good effectiveness of lemongrass and cinnamon EOs. Lemongrass and cinnamon EOs were tested in a laboratory-scale yogurt production to confirm the effectiveness of these EOs *in vivo*, against three different species belonging to *Debaryomyces hansenii*, *Candida pararugosa* and *Yarrowia deformans*, previously inoculated in the yogurt. Cinnamon EO has demonstrated an antimicrobial activity that leads to an incomplete milk fermentation, inhibiting the growth of *L. bulgaricus* and *S. thermophilus* starter strains. This result demonstrates that cinnamon

EO is not applicable as a preservative in yogurt; it could be used as preservative against yeast spoilage in other foods or beverages. The antifungal activity of lemongrass EO has been confirmed *in vivo* only against *D. hansenii* and *Y. deformans* isolates. Therefore, the effectiveness lemongrass EO antifungal activity is species-dependent. However, lemongrass EO has a negative impact on the final product's taste. Further researches could be carried out to amplify the potential application of lemongrass EO in other foods and beverages production. Overall, the data obtained from the two studies highlight the importance to increasing the study about antifungal activity of EOs. Indeed, the results obtained from cinnamon, lemongrass and orange EOs against different yeast spoilage species are encouraging, but their impact and their applicability in food matrices remains to be verified *in vivo* because the pronounced aromatic notes associated with these two EOs could limit the possible range of applications.

Overall, the data obtained in the different studies have highlighted the importance of further analyses to increase the knowledge about yeast's role of in production of foods and beverages with health benefits. Up to now, encouraging data have been acquired on the possibility of producing a beer enriched in B_3 vitamin, thanks to the releasing of this molecule by *Saccharomyces cerevisiae*'s strains. Further analyses are necessary to increase the knowledge on kefir drinks and in particular on kefir produced through the backslopping method.

Detection, prevention and control of spoilage events caused by yeasts are important due to their increasing. Considering the economic losses, development of technique to detect and quantify these microorganisms in a quick and easy way could be useful for this purpose. At last, the studies carried on the evaluation of EOs' antifungal activity have led to encouraging results about the possibility of using EOs as preservatives in foods and beverage formulation. However, further analyses are necessary to verify this potential role as preservatives against yeast spoilage in the food industry.

Appendix

		ESSENTIAL OIL						
Isolate code	Species	Lime	Ginger	Lemongrass	Mandarin	Cinnamon	Orange	Lemon
1	***Yarrowia lipolytica***	0 ±0.0 **b**	0±0.0 **b**	39 ± 1.4 **a**	0±0.0 **b**	43±7.1 **a**	10±0.0 **b**	4±0.0 **b**
6	***Yarrowia lipolytica***	8±0.0 **b**	0±0.0 **b**	38±0.0 **a**	0±0.0 **b**	42±14.1 **a**	11±1.4 **b**	4±5.7 **b**
9	***Candida sake***	4±0.0 **c**	0±0.0 **c**	54±0.0 **a**	8±0.0 **c**	49±4.2 **a**	25±4.2 **b**	0±0.0 **c**
10	***Candida sake***	8±0.0 **c**	0±0.0 **c**	56±0.0 **a**	8±0.0 **bc**	55±7.1 **a**	14±2.8 **b**	9±1.4 **bc**
12	***Debaryomyces hansenii***	8±0.0 **c**	0±0.0 **b**	52±0.0 **a**	4±5.7 **b**	43±7.1 **a**	11±1.4 **b**	8±0.0 **b**
13	***Debaryomyces hansenii***	8±0.0 **b**	0±0.0 **b**	44±2.8 **a**	0±0.0 **b**	39±1.4 **a**	10±2.8 **b**	5±7.1 **b**
14	***Debaryomyces hansenii***	8±0.0 **b**	0±0.0 **c**	47±4.2 **a**	0±0.0 **c**	35±1.4 **b**	10±0.0 **c**	4±5.7 **c**
16	***Yarrowia lipolytica***	0±0.0 **c**	0±0.0 **c**	40±5.7 **a**	0±0.0 **c**	38±0.0 **a**	10±0.0 **b**	0±0.0 **c**
17	***Yarrowia lipolytica***	0±0.0 **b**	0±0.0 **b**	40±5.7 **a**	0±0.0 **b**	42±5.7 **a**	10±0.0 **b**	0±0.0 **b**
19	***Candida parapsilosis***	5±7.0 **bc**	0±0.0 **c**	36±0.0 **a**	0±0.0 **c**	38±2.8 **a**	14±0.0 **b**	0±0.0 **c**
20	***Debaryomyces hansenii***	9±1.4 **bc**	0±0.0 **c**	39±4.2 **a**	0±0.0 **c**	45±1.4 **a**	12±2.8 **b**	4±5.7 **bc**
22	***Debaryomyces hansenii***	10±2.8 **b**	0±0.0 **b**	44±2.8 **a**	0±0.0 **b**	40±8.5 **a**	12±2.8 **b**	8±0.0 **b**
24	***Clavispora lusitaniae***	12±0.0 **cd**	0±0.0 **e**	29±1.4 **b**	8±0.0 **d**	38±0.0 **a**	14±2.8 **c**	8±0.0 **d**
26	***Debaryomyces hansenii***	8±0.0 **c**	0±0.0 **d**	49±4.2 **a**	8±0.0 **c**	38±0.0 **b**	11±1.4 **c**	11±1.4 **c**
28	***Yarrowia lipolytica***	4±5.6 **bc**	0±0.0 **c**	39±1.4 **a**	0±0.0 **c**	40±0.0 **a**	10±0.0 **b**	0±0.0 **c**
31	***Yarrowia lipolytica***	0±0.0 **b**	0±0.0 **b**	38±2.8 **a**	0±0.0 **b**	38±0.0 **a**	9±1.4 **b**	4±5.7 **b**
33	***Candida sake***	10±0.0 **bc**	0±0.0 **c**	50±2.8 **a**	9±1.4 **bc**	29±15.6 **ab**	17±1.4 **bc**	9±1.4 **bc**
34	***Debaryomyces hansenii***	8±0.0 **b**	0±0.0 **b**	45±4.2 **a**	0±0.0 **b**	36±2.8 **a**	10±0.0 **b**	4±5.7 **b**
35	***Debaryomyces hansenii***	10±0.0 **cd**	4±5.7 **d**	71±7.1 **a**	8±0.0 **d**	41±4.2 **b**	24±0.0 **c**	9±1.4 **cd**
38	***Candida intermedia***	10±0.0 **bc**	0±0.0 **c**	43±4.2 **a**	8±0.0 **bc**	46±0.0 **a**	12±0.0 **b**	4±5.7 **bc**
42	***Clavispora lusitaniae***	10±2.8 **bc**	0±0.0 **c**	42±11.3 **a**	0±0.0 **c**	30±14.1 **ab**	15±1.4 **abc**	0±0.0 **c**
43	***Debaryomyces hansenii***	8±0.0 **bc**	0±0.0 **c**	48±0.0 **a**	0±0.0 **c**	26±11.3 **b**	9±1.4 **bc**	4±5.7 **c**
44	***Debaryomyces hansenii***	8±0.0 **c**	0±0.0 **d**	47±1.4 **a**	9±1.4 **c**	31±1.4 **b**	10±0.0 **c**	9±1.4 **c**
45	***Debaryomyces hansenii***	10±2.8 **c**	0±0.0 **d**	46±0.0 **a**	0±0.0 **d**	34±2.8 **b**	11±1.4 **c**	9±1.4 **c**
46	***Yarrowia lipolytica***	10±0.0**abc**	0±0.0 **c**	29±15.6 **ab**	0±0.0 **c**	34±2.8 **a**	10±0.0 **abc**	4±5.7 **bc**
47	***Yarrowia lipolytica***	4±5.7 **bc**	0±0.0 **c**	26.5±16.3 **ab**	0±0.0 **c**	34±2.8 **a**	9±01.4 **abc**	0±0.0 **c**
50	***Rhodotorula babjevae***	12±0.0 **c**	0±0.0 **e**	35±1.4 **b**	8±0.0 **d**	44±0.0 **a**	13±1.4 **c**	8±0.0 **d**
51	***Rhodotorula babjevae***	8±0.0 **b**	0±0.0 **b**	34±0.0 **a**	9±1.4 **b**	32±8.5 **a**	10±0.0 **b**	4±5.7 **b**
52	***Debaryomyces hansenii***	8±0.0 **c**	0±0.0 **c**	45±1.4 **a**	4±5.7 **c**	32±2.8 **b**	9±1.4 **c**	8±0.0 **c**
53	***Debaryomyces hansenii***	8±0.0 **b**	4±5.7 **b**	50±2.8 **a**	4±5.7 **b**	39±4.2 **a**	12±0.0 **b**	10±0.0 **b**
54	***Yarrowia lipolytica***	0±0.0 **b**	0±0.0 **b**	38±5.7 **a**	4±5.7 **b**	35±1.4 **a**	9±1.4 **b**	0±0.0 **b**
55	***Yarrowia lipolytica***	0±0.0 **c**	0±0.0 **c**	38±2.8 **a**	0±0.0 **c**	41±4.2 **a**	9±1.4 **b**	0±0.0 **c**

56	***Debaryomyces hansenii***	10±0.0 **d**	0±0.0 **e**	48±0.0 **a**	0±0.0 **e**	41±1.4 **b**	13±1.4 **c**	8±0.0 **d**
57	***Yarrowia deformans***	10±0.0 **c**	0±0.0 **d**	27±1.4 **b**	0±0.0 **d**	42± 0.0 **a**	4±5.7 **cd**	0±0.0 **d**
59	***Candida pararugosa***	17±1.4 **bc**	8±0.0 **cd**	49±4.2 **a**	4±5.7 **cd**	42± 2.8 **a**	23±4.2 **b**	0±0.0 **d**
60	***Candida pararugosa***	9±1.4 **c**	0±0.0 **c**	55±4.2 **a**	0±0.0 **c**	44± 5.7 **ab**	30±2.8 **b**	4±5.7 **c**
61	***Kluyveromyces lactis***	11±1.4 **bc**	0±0.0 **c**	49±4.2 **a**	4±5.7 **c**	47±4.2 **a**	20±2.8 **b**	9±1.4 **bc**
62	***Yarrowia deformans***	9±1.4 **b**	0±0.0 **c**	34±0.0 **a**	0±0.0 **c**	31± 1.4 **a**	10±0.0 **b**	4±5.7 **bc**
63	***Yarrowia deformans***	9±1.4 **b**	0±0.0 **c**	31±1.4 **a**	0±0.0 **c**	30±0.0 **a**	4±5.7 **bc**	0±0.0 **c**
64	***Debaryomyces hansenii***	8±0.0 **b**	0±0.0 **b**	40±5.7 **a**	4±5.7 **b**	33±1.4 **a**	10±0.0 **b**	4±5.7 **b**
65	***Debaryomyces hansenii***	8±0.0 **b**	0±0.0 **b**	38±2.8 **a**	4±5.7 **b**	32±2.8 **a**	10±0.0 **b**	4±5.7 **b**
66	***Yarrowia deformans***	0±0.0 **b**	0±0.0 **b**	35±7.1 **a**	0±0.0 **b**	33±1.4 **a**	8±0.0 **b**	0±0.0 **b**
67	***Yarrowia deformans***	0±0.0 **b**	0±0.0 **b**	37±9.9 **a**	0±0.0 **b**	35±4.2 **a**	9±1.4 **b**	11±1.4 **b**
69	***Candida pararugosa***	8±0.0 **bc**	0±0.0 **c**	46±14.1 **a**	0±0.0 **c**	44±2.8 **a**	28±2.8 **b**	4±5.7 **c**
70	***Yarrowia lipolytica***	0±0.0 **b**	0±0.0 **b**	32±8.5 **a**	0±0.0 **b**	36±2.8 **a**	10±0.0 **b**	5±7.1 **b**
71	***Yarrowia lipolytica***	0±0.0 **b**	0±0.0 **b**	41±9.9 **a**	4±5.7 **b**	36±2.8 **a**	9±1.4 **b**	5±7.1 **b**
72	***Rhodotorula glutinis***	0±0.0 **d**	0±0.0 **d**	34±0.0 **b**	0±0.0 **d**	40±2.8 **a**	13±1.4 **c**	0±0.0 **d**
73	***Rhodotorula diobovata***	8±0.0 **b**	8±0.0 **b**	42±2.8 **a**	8±0.0 **b**	47±1.4 **a**	14±2.8 **b**	5±7.1 **b**
74	***Candida aaseri***	10±0.0 **bc**	0±0.0 **c**	38±5.7 **a**	4±5.7 **bc**	43±1.4 **a**	16±0.0 **b**	9±1.4 **bc**
75	***Yarrowia lipolytica***	0±0.0 **b**	0±0.0 **b**	36±0.0 **a**	4±5.7 **b**	36±8.5 **a**	8±0.0 **b**	4±5.7 **b**
76	***Yarrowia lipolytica***	0±0.0 **d**	0±0.0 **d**	41±1.4 **b**	0±0.0 **d**	50±5.7 **a**	9±1.4 **c**	0±0.0 **d**
78	***Yarrowia lipolytica***	0±0.0 **c**	0±0.0 **c**	39±4.2 **a**	0±0.0 **c**	33±4.2 **a**	10±2.8 **b**	0±0.0 **c**
79	***Yarrowia lipolytica***	0±0.0 **b**	0±0.0 **b**	37±4.2 **a**	0±0.0 **b**	33±4.2 **a**	11±1.4 **b**	4±5.7 **b**
80	***Yarrowia lipolytica***	0±0.0 **b**	0±0.0 **b**	29±1.4 **a**	0±0.0 **b**	37±4.2 **a**	10±0.0 **b**	5±7.1 **b**
81	***Candida parapsilosis***	8±0.0 **bc**	0±0.0 **c**	49±7.1 **a**	8±0.0 **bc**	44±2.8 **a**	13±1.4 **b**	12±0.0 **b**
82	***Debaryomyces hansenii***	8±0.0 **cd**	0±0.0 **d**	49±4.2 **a**	0±0.0 **d**	30±2.8 **b**	13±4.2 **d**	0±0.0 **d**
83	***Debaryomyces hansenii***	8±0.0 **bc**	0±0.0 **c**	37±4.2 **a**	8±0.0 **bc**	39± 4.2 **a**	14±0.0 **b**	9±1.4 **bc**
84	***Candida sake***	10±0.0 **c**	0±0.0 **d**	54±2.8 **a**	9±1.4 **c**	42±0.0 **a**	14±0.0 **c**	10±2.8 **c**
85	***Debaryomyces hansenii***	8±0.0 **b**	0±0.0 **d**	41±1.4 **a**	0±0.0 **d**	39±1.4 **a**	14±0.0 **b**	8±0.0 **c**
86	***Yarrowia deformans***	10±2.8 **b**	0±0.0 **b**	47±7.1 **a**	9±1.4 **b**	44±0.0 **a**	12±2.8 **b**	5±7.1 **b**
87	***Yarrowia lipolytica***	9±1.4 **bc**	0±0.0 **c**	33±15.6 **ab**	0±0.0 **c**	35±1.4 **a**	9±1.4 **bc**	5± 7.1 **c**
88	***Yarrowia lipolytica***	9±1.4 **bc**	0±0.0 **c**	31±18.4 **ab**	0±0.0 **c**	44±8.5 **a**	10±0.0 **bc**	0±0.0 **c**
89	***Yarrowia lipolytica***	9±1.4 **bc**	0±0.0 **c**	22±8.5 **b**	0±0.0 **c**	41±4.2 **a**	8±0.0 **bc**	0±0.0 **c**
90	***Yarrowia lipolytica***	0±0.0 **c**	0±0.0 **c**	28±8.5 **ab**	4±5.7 **c**	36±2.8 **a**	11±1.4 **bc**	5±7.1 **c**
91	***Yarrowia lipolytica***	8±0.0 **c**	0±0.0 **d**	31±1.4 **b**	0±0.0 **d**	48±2.8 **a**	11±1.4 **c**	0±0.0 **d**
92	***Debaryomyces hansenii***	8±0.0 **c**	0±0.0 **c**	51±4.2 **a**	4±5.7 **c**	30±2.8 **b**	10±0.0 **c**	0±0.0 **c**
93	***Debaryomyces subglobosus***	9±1.4 **cd**	0±0.0 **d**	56±2.8 **a**	4±5.7 **cd**	38±2.8 **b**	12±0.0 **c**	0±0.0 **d**
94	***Debaryomyces hansenii***	8±0.0 **bc**	0±0.0 **c**	48±0.0 **a**	4±5.7 **bc**	43±1.4 **a**	13±1.4 **b**	9±1.4 **bc**
95	***Yarrowia lipolytica***	0±0.0 **d**	0±0.0 **d**	28±2.8 **b**	0±0.0 **d**	34±4.2 **a**	11±1.4 **c**	0±0.0 **d**
96	***Yarrowia lipolytica***	8±0.0 **bc**	0±0.0 **b**	40±0.0 **a**	0±0.0 **b**	40±5.7 **a**	11±1.4 **b**	0±0.0 **c**
97	***Yarrowia lipolytica***	0±0.0 **b**	0±0.0 **b**	38±5.7 **a**	0±0.0 **b**	36±5.7 **a**	10±0.0 **b**	4±5.7 **b**
98	***Yarrowia lipolytica***	0±0.0 **b**	0±0.0 **b**	38±5.7 **a**	0±0.0 **b**	41±4.2 **a**	8±0.0 **b**	4±5.7 **b**
99	***Yarrowia lipolytica***	8±0.0 **c**	0±0.0 **d**	31±1.4 **b**	0±0.0 **d**	35±1.4 **a**	8±0.0 **c**	0±0.0 **d**

100	***Yarrowia lipolytica***	9±1.4 **b**	0±0.0 **c**	35±1.4 **a**	0±0.0 **c**	33±1.4 **a**	8±0.0 **b**	0±0.0 **c**

Supplementary table 1. Results of antifungal activity (growth inhibition zone) of EOs tested on 74 yeast spoilage isolates collected from yogurt. Averages and standard deviation of growth inhibition zone are shown for each isolate. The results of the effectiveness of the EOs, expressed in millimetres, are submitted to statistical analysis of one way ANOVA. Significantly differences were considered for $P < 0.05$.