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Frontiers and New Trends in the Science of Fermented Food and Beverages

Edited by Rosa Lidia Solís-Oviedo and Ángel de la Cruz Pech-Canul

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Meet the editors

Dr. Solís-Oviedo is a researcher belonging to the National System of Researchers (SNI) from the Mexican National Council for Sciences and Technology (CONACyT). She is a member of the National Registry of Accredited Science Reviewers (RCEA-CONACyT) at the Biology/Chemistry Division. Her current interests are the exploitation of industrial properties of beneficial strains, specifically in food fermentations, and the production of chemical commodities from renewable resources. Dr. Solís-Oviedo holds a PhD in Biomedical Sciences and a BSc in Biochemical Engineering. She held a postdoctoral position at the University of Nottingham, where she gained experience working with modern genetic tools and microbial biotechnology aspects for the production of metabolites of industrial interest from industrial wastes, as well as on the training of human resources.

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Contents

Preface

From time immemorial fermented foods have undoubtedly contributed to human health and well-being, and consequently to the progress of modern societies. The earliest archaeological evidence for the preparation of recipes was found in the Middle East. However, archaeological evidence, such as dedicated utensils for fermentation, are even older than these recipes. Among many fermented foods, both beer and bread flourished in the ancient Egyptian civilization and were considered as basic food items for their citizens. Historically, the preparation of bread, beer, and wine has been popular across Europe for centuries. Nevertheless, natives from many ancient cultures worldwide still conduct a wide variety of food fermentations using deep-rooted recipes and processes.

For centuries, the art of fermented foods was mastered by pure empirical observation, for example, through the domestication of yeast strains. Within the last four centuries, scientific research has started to unravel many aspects of the biological process. Hence, it has contributed to the improvement of many industrial processes. Nowadays, it is well known that fermentations are conducted by microorganisms. Therefore, fermented foods can be categorized according to either the primary metabolic product or the microorganism involved in the biological process. The food used as substrate in the fermentation process can also be used to describe fermented foods.

The modern food industry allows massive production of fermented products. However, somehow it has limited the diversification of fermented foods available in the market. Fortunately, several traditional fermented foods have recently regained attention mainly due to their nutritional values. Through our journey in the research field, we have always been attracted to the development of scientific research around autochthonous fermented foods and their cultural roots. These unique ferments are a wide-open window for new biodiversity. Furthermore, they are a natural repository of novel biological processes and biomolecules that will positively impact on many application fields from health, to food, to materials. Despite this, we find that many of these exciting results are regularly scattered and hard to find, because they are regularly spread in conference reports or in local journals. Furthermore, many of these reports remain in their original language and consequently are out of sight. Thus, most of the research progresses in this area are being hampered by the lack of publications in internationally recognized journals or books. Unfortunately, this has been a common issue, especially in developing countries where scientific research is consciously considered as a basis for economic growth.

The main purpose of this book is to provide useful and novel information for readers regarding fermented foods. The content aims to expand the knowledge of fermented foods emphasizing their research progresses, new trends, and diversity. The book also aims to promote the interest of readers in this particular area of research. Chapters focus on many different research disciplines. For example, ethanol from fermentations is presented, including its social implications. Research progresses on tea-derived beverages are also presented along with advances in prebiotic and

symbiotic products. The book also includes exciting examples of African fermented foods. On the other hand, two divergent approaches to improve fermentation by the controlled inoculation of microorganisms are discussed, contrasting their strengths and weaknesses. Finally, research on genetics is presented as a valuable tool to deeply understand the basics of fermentation and its perspectives for the progress of the modern food industry. We were honoured and privileged by the valuable participation of researchers worldwide, such as from many African countries, Australia, Singapore, Romania, Argentina, Peru, and Mexico. The eclectic participation allowed us to enrich the outlook on the diversity of fermented foods and their potential impacts on human well-being.

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Chapter 1

Alcoholic Beverages and Human Health: An Overview

Oladipo Iyabo Christianah

Abstract

Production of alcohol is by fermentation of yeast, sugar, and starches. The consumption of which may be associated with some underlining risk factor depending on the quantity consumed per time. Alcohol can be consumed raw or by mixing in beverages, and whenever an alcoholic beverage is consumed, it can take about an hour for the body to metabolize one-eight of an imperial gallon. The level of the blood alcohol is increased when the quantity of alcohol consumed exceeds the normal dose which the body could metabolize, and then intoxication sets it. The higher the blood alcohol concentration, the higher the risk of diseases associated with the liver, kidney, and pancreas and the abundance of free radicals in the body system. Excessive use of alcohol can lead to alcoholism or alcohol dependence. Withdrawal from which can be life-threatening. Disulfiram, naltrexone, and acamprosate are the three approved oral medications for the treatment of alcoholism or alcohol dependence. On the other hand, moderate consumption of alcohol or red wines has been confirmed to be beneficial to human health particularly because of the antioxidant properties it confers.

Keywords: alcoholism, alcohol dependence, alcohol addiction, alcohol withdrawal, signs and symptoms

1. Introduction

Alcohol are organic compounds identified by one or more hydroxyl ($^{-}$OH) groups attached to a carbon atom of an alkyl group [1]. Alcohol is often considered as organic derivatives of water in which one of the hydrogen atoms has been replaced by an alkyl group, typically represented by R in organic structures. There are different types of alcohol: propyl, methyl, ethyl, and butyl alcohol, but for the production of alcoholic beverages, ethyl alcohol (ethanol) is the type used, while death or blindness could result if the other three are consumed at all [2]. Ethanol is normally produced from the fermentation of yeast, sugar, and starches [3]. It can also be consumed mixed in beverages or in its raw form, but the end product after digestion by the body is formaldehyde, which is deleterious to the body and also the cause of alcohol poisoning. Liver cirrhosis, gastric ulcers, gastritis, fatty liver, alcoholic hepatitis, and pancreatitis are examples of some disorders resulting from alcohol intoxication. Moderate consumption of alcohol may have some health benefits that could prolong life, but when taken in excess or as an escape route from problems, then abuse or alcoholism is inevitable.

Abuse of alcohol usually leads to alcohol use disorder also known as alcoholism or alcohol addiction which can lead to a violent behavior. Addiction to alcohol refers

to physical and psychological dependency on alcohol to the point that tolerance is built up to alcohol [4]. Furthermore, once the body becomes adapted to excessive consumption of alcohol, an abrupt discontinuation leads to withdrawal symptoms which can be a threat to life and the symptoms normally includes hallucination, tremors, convulsions, anxiety, etc. Alcohol addiction may also result in the following: restlessness, depression, erratic behavior, decreased involvement in extracurricular activities, loss of interest in work or school, lack of interest in relationships, preoccupation with drinking, inability to control drinking, and violent behavior. While, nonalcoholic beverage refers to any drink that contains no alcohol or which contains less than 0.5% alcohol by volume and examples are fruit juices, mineral water, hot drinks etc.

2. Alcohol and alcoholic beverages

There are different types of alcohol based on their uses; some are used as solvents, antifreeze solutions, e.g., isopropyl and methyl alcohol, paint removers in chemistry laboratories and chemical factories, and as nail polish remover and cleansing solvent, for example, methylated spirit for domestic use [5]. There are different types of alcohol depending on the number of carbon atoms present and the position of the OH bond in the formula, but the most common alcohol is ethanol (CH_3CH_2OH or C_2H_5OH). During the process called fermentation, alcohol is produced. Fermentation of yeast usually results into sugar breaking down into carbon dioxide and alcohol. But carbon dioxide is taking off the process through gas bubbles leaving a mixture of water and ethanol. Sugar fermentation to alcohol can be used in different applications especially in the production of alcoholic beverages, for example, the extracts of grapes and barley are fermented in the wine and beer industry to produce alcoholic beverages. Any drink or beverage that contains ethanol is normally referred to as alcoholic beverage and are either produced by fermentation or distillation. There are different types of alcoholic and nonalcoholic beverages. Alcoholic drinks can be classified into five categories: (i) Wine, i.e., still, sparkling, fortified, or aromatized; (ii) beer, i.e., ales, lagers, and stouts; (iii) cider and perry; (iv) distilled spirits, i.e., vodka, gin, rum, whiskey, brandy, and others; and (v) liqueurs, i.e., flavored with fruit, citrus, herb, kernel, flower, cream, and berry. And nonalcoholic drinks can be classified into four categories: (i) Hot drinks, i.e., tea, coffee, and chocolate; (ii) fruit juices; (iii) mineral waters; and (iv) cordials/syrups.

3. Alcohol and human health

Alcohol has a complex mode of action and is majorly a depressant on the central nervous system, and the brain is affected in the process. It activates the release of the chief inhibitory neurotransmitter in the central nervous system by binding to GABA (gamma-aminobutyric acid) receptors in the brain. Absorption of alcohol which takes place from the intestine is reduced by fatty foods, and alcohol is distributed into body water. Alcoholic beverages contain ethyl alcohol, and research has shown that utilization of alcohol has here and now mental and physiological impacts on the consumer [6, 7]. Diverse absorption of alcohol in the human body affects a man, and the impacts of alcohol rely upon the quantity an individual has consumed, the level of alcohol in the mixed drinks and the time length that the utilization occurred, the measure of sustenance eaten and whether an individual has taken other remedies, and over-the-counter or road drugs, among different

elements. Alcohol in carbonated drinks is absorbed faster than alcohol in noncarbonated drinks [6, 7].

Blood alcohol concentration (BAC) is dependent on amount and time range of alcohol utilization, muscle to fat ratio and weight, and nourishment impacts. Overabundance utilization of alcohol on an unfilled stomach over a brief timeframe range as a rule results to higher BAC. Drinking enough to cause a blood alcohol concentration (BAC) of 0.03–0.12% ordinarily causes a general perking up and conceivable happiness; expanded self-assurance and amiability; diminished nervousness; a flushed, red appearance in the face; impeded judgment; and fine muscle coordination. A BAC from 0.09 to 0.25% causes torpidity, sedation, adjust issues, and obscured vision. A BAC from 0.18 to 0.30% causes significant disarray, hindered discourse (e.g., slurred discourse), unsteadiness, and spewing [6, 7]. A BAC from 0.25 to 0.40% causes trance, obviousness, anterograde amnesia, retching (passing may happen because of inward breath of regurgitation (pneumonic goal) while oblivious), and respiratory melancholy (possibly dangerous). A BAC from 0.35 to 0.80% causes a state of unconsciousness (obliviousness), hazardous respiratory misery, and potentially deadly alcohol harming. Likewise with every mixed drink, drinking while at the same time driving, working an airship, or substantial hardware builds the danger of a mishap; numerous nations have punishments against driving while intoxicated [8].

Metabolization of 90% of consumed alcohol takes place in the liver; alcohol is converted to acetaldehyde by dehydrogenase (which is a sympathomimetic toxin responsible for "hangover"). Then, acetaldehyde is metabolized to acetic acid by aldehyde dehydrogenase and finally to carbon dioxide and water. Alcohol can then be excreted from the body through the sweat, lungs, and urine.

The regular consumption of alcohol, despite the bad effects it has on human's life, is called addiction or alcoholism. The causes of alcoholism usually include one or more of the following: peer pressure, usage of alcohol as remedy for mental ill health, alcoholism gene, influence of an alcoholic parent, etc. The abuse of alcohol results in over a million deaths in teenagers and young adults due to accidents every year all over the world [9]. Alcohol abuse occurs when all or one of the following happens: (i) Excess alcohol consumption in a social gathering, (ii) drinking and driving, (iii) alcohol consumption throughout the day, (iv) alcohol consumption to feel high, and (v) the need to drink alcohol every day.

Cognitive ability is impaired due to alcohol consumption, for example, in occasional and moderate drinkers the following occurs: memory impairment, blackout, recklessness, and impaired decision-making. While the following results in heavy and/or chronic drinkers: diminished brain size, inability to think abstractly, loss of visuospatial abilities, Wernicke-Korsakoff syndrome, loss of memory, and poor attention span. There are five different types of alcoholics which are (i) young adult subtype which includes young adults whose family have no history of alcoholism or mental ill health; (ii) young antisocial subtype, also includes young adult with a family history of alcoholism, mental ill health, and other addictions; (iii) functional subtype, this includes gainfully employed and successful middle-aged with a supportive family; they have a family history of alcoholism while some of them may have a history of depression (iv) intermediate familial subtype, these are middle-aged people with prior episode of depression and a family history of alcoholism, (v) chronic severe subtype are the middle-aged people with family histories of alcoholism, mental ill health, and other addictions [10].

3.1 Toxicity of alcohol

The toxic metabolic effects of alcohol are due both to its direct action when few percent of it enter the bloodstream from the stomach and that of its first metabolite

called acetaldehyde (belongs to the same chemical family with formaldehyde) which is highly toxic to man [11]. Alcohol increases the rate of generation of free radicals in the body system and also inhibits the antioxidant levels thus inducing oxidative stress [6]. The excessive consumption of alcohol can also result in immunodeficiency and immunosuppressant thus exposing such individual to different kinds of infections. Research has shown that the consumption of alcohol is one of the causes of acute illness and chronic diseases throughout the world today [7].

3.2 Alcohol and pregnancy

Consumption of alcohol during pregnancy can have deleterious effects on the fetus not only in the first trimester but also throughout pregnancy as alcohol can move by the umbilical cord and placenta from the mother's bloodstream to the fetus. Alcohol consumption can cause havoc on the pregnancy before a woman is aware of the pregnancy. The havoc that can be caused on pregnancy includes miscarriage, birth deformities, retarded growth, and mental defects [2]. Excessive consumption of alcohol in pregnant women results in fetal alcohol syndrome (FAS) or fetal alcohol spectrum disorders (FASD) characterized with irreversible mental and physical changes to the baby. Fetal alcohol syndrome (FAS) may cause skeletal and facial abnormalities, growth retardation, mental disorders, and heart defects, while in fetal alcohol spectrum disorders (FASD), hyperactivity, life-long learning disabilities, poor attention span, speech or language delays, poor memory, and other disorders may result [12–14]. It is generally advised that drinking should be completely avoided by pregnant women.

3.3 Alcohol's effects on the body

Frequent alcohol consumption can be deleterious to human's health by affecting the following: (i) Brain: the communicative pathways of the brain are disrupted which may result in mood swing, lack of coordination, and coherence. (ii) Heart: it may also affect the heart by causing cardiomyopathy, arrhythmias, stroke, and high blood pressure. (iii) Liver: different types of problems may occur in the liver including liver inflammations, cirrhosis, alcoholic hepatitis, fibrosis and steatosis, or fatty liver [15]. (iv) Pancreas: heavy consumption of alcohol may cause the blood vessels in the pancreas to be swollen and inflamed thereby preventing proper digestion [16]. (v) Skin: abuse and consumption of alcohol can cause a variety of skin disorders [17]. Thermoregulation of the body also results in skin vasodilation and sweating [18]. (vi) Cancer: heavy drinkers stand the chance of developing cancers like cancer of the mouth, esophagus, throat, liver, and breast [16, 19]. Alcohol meddles with folate assimilation and function in the body, which may be one way alcohol can build danger of causing specific cancers. (vii) Immune system: alcoholism results in immune system being compromised, and the body is easily prone to diseases like pneumonia and tuberculosis.

4. Signs and symptoms of alcohol abuse and addiction

Some of the signs and symptoms of alcoholism includes the following in most adults: stomach pains, vomiting or nausea, redness of the face during or after periods of consumption, delayed reflexes, loss of consciousness or blacking out, slurred or incoherent speech, poor balance, and clumsiness. Alcoholics reach a level that the breathing is affected due to depression of the respiratory system which sometimes results into death. Some alcoholics become dependent on alcohol

up to a point that their health, work, and relationship are neglected [20]. Signs of alcohol abuse include the following: insomnia, anger, loss of control and attention, etc. Alcoholism if untreated results in addiction identified by dependency. Signs of alcohol dependence are tolerance to increase in alcohol consumption, hangover, unsuccessful attempt to reduce consumption, alcohol withdrawal symptoms when alcohol is not consumed, etc. Seizures, extreme agitation or anxiety, hallucinations, nausea or vomiting, tremors, convulsions, uncontrolled shaking of the hands, persistent insomnia, and profuse sweating are some of the symptoms of withdrawal from alcohol indicative of advanced stage of addiction and should not be handled lightly [11]. Medical detoxification is usually the treatment given to alcoholism.

5. Side effects of alcohol withdrawal

Withdrawal from alcohol normally kicks off between 6 and 24 hours after the last drink, and withdrawal has been categorized into three stages according to their severity: (i) stage one which is the mild stage involves tremors, abdominal pain and/or vomiting, foggy thinking, anxiety, insomnia, nausea, loss of appetite, fatigue, depression, mood swings, and heart palpitations; (ii) stage two which is the moderate stage also involves mental confusion; irregular heart rate; profuse sweating; irritability; increased blood pressure, body temperature, and respiration; and heightened mood disturbances; and (iii) stage three which is the severe stage is often called the delirium tremens. It is characterized by fever, agitation, severe confusion, hallucination, and seizures. Delirium tremens often occur in about 3–5% alcohol withdrawal individuals. It normally kicks off without warning at 24 or 48 hours after alcohol leaves the bloodstream. Withdrawal from alcohol is influenced by factors such as stress level, family history of addiction, co-occurring mental ill health, childhood trauma, drinking duration, and quantity consumed. It can also be influenced by combined use of alcohol and other drugs thereby increasing the potential for dangers and other side effects. Withdrawal from alcohol can in some cases cause the death of an addict as the central nervous system and the brain are normally affected by continuous suppression by alcohol for a long period of time.

Withdrawal from alcohol has no definite timeline, but a general timeline has been detailed as follows: the first stage normally begins 8 hours after the first drink, while the second and third stages occur rapidly, and symptoms peak between 24 and 72 hours. Symptoms may then start to wear off 5–7 days later, but psychological symptoms may be prolonged for several weeks [21].

Physical symptoms are monitored and controlled up to a stable point during detoxification which is often achieved by medications. Restoration of the natural order to the overactivity of the central nervous system can be accomplished by using benzodiazepines during alcohol detoxification. Dangerous side effects of alcohol withdrawal can be avoided by gradual and controlled weaning of alcohol out of the body.

5.1 Managing withdrawal symptoms

Thought of suicide, anxiety, and depression can be controlled with medications alongside therapy and counseling. Disulfiram, naltrexone, and acamprosate are the three approved medications used to control alcohol cravings in the treatment of alcohol withdrawal. Naltrexone operates by blocking the opioid receptors in the brain, thereby reducing cravings for drinking, while long-term withdrawal symptoms are taken care of by acamprosate. Disulfiram makes drinking undesirable by making people sick if they drink. Another promising medication in the treatment of alcohol use disorders is topiramate [2].

6. Beneficial effects of alcohol

Red wines have been known to have antioxidant properties because they contain substances like resveratrol (polyphenol) and flavonoids which confer cardioprotective effects to the heart. Antioxidants also protect against artery damage by increasing levels of high density lipoprotein (HDL). So, moderate consumption of alcohol or red wines has been confirmed to be beneficial to human health. But it should not be used as an excuse to start drinking as it can be addictive or cause other health problems [2].

Also, it is generally known that red wine contains the most resveratrol, the antioxidant found in grape skin that may improve the health of the heart by counteracting blood vessel harm and also diminishing LDL cholesterol. Additionally, the flavonoids contained in red wine are another vital cancer prevention agent. It must then be noted that most research carried out have been on animals, and the measure of wine to be taken by an individual to level with a similar sum given to mice would neutralize any of the assumed advantages. Likewise, the 129 calories contained in a glass can accumulate, regardless of whether an individual is just drinking one daily [22]. Red wine also contains histamine that can cause headache assaults or different kinds of manifestations that happen from a histamine prejudice.

7. Treatment of alcohol abuse and alcoholism

Alcoholism treatment involves serious medical, family, and social support, while interventions like group support, individual counseling, stepped therapy, and medications are usually advocated for alcohol dependence. Three approved medications for medical treatments of alcohol dependence are naltrexone (Depade, ReVia), disulfiram (Antabuse), and acamprosate (Campral) and an injectable long-acting form of naltrexone (Vivitrol). The medication may reduce relapses and drinking and may result to total recovery and abstinence from alcohol. Naltrexone and acamprosate have been recommended as treatment alternative for alcohol dependence together with behavioral therapy in a review, but disulfiram has not been recommended for routine use in primary care as proof of its ability to increase abstinence rates or decrease relapse rates or cravings compared with placebo as not been shown [4].

8. Alcohol detoxification

Alcohol detoxification is the process by which the system of a heavy drinker is returned to normal after prolonged period of alcohol abuse. It normally involves medical treatment and counseling. The detoxification process involves three steps: (i) Step one (intake) involves a comprehensive review of drug, psychiatric, and medical histories of patients to fully understand the situation, (ii) step two (stabilization) which involves medical and psychological therapies to help a patient reach a balance of mind and body, and (iii) step three (medication) which involves medications that mimic the effects of alcohol to mitigate withdrawal symptoms. Some of the unpleasant side effects of detoxification may include nausea, insomnia, mood swing, muscle weakness, and nervousness, while kidney or liver dysfunction, seizures, extreme nausea, aspiration pneumonia, heart arrhythmias, fever, and hallucinations are examples of some of the severe side effects of alcohol detoxification. Medications are used to avoid physiological upsets and system imbalance during detoxification. Benzodiazepines are used to reduce alcohol withdrawal symptoms

and also prevent alcohol withdrawal seizures which are the most common causes of fatality in alcohol withdrawal [23].

9. Nonalcoholic drink

Beverage without an alcohol is usually referred to as a nonalcoholic drink. There are different varieties of nonalcoholic beverages; examples are soft drinks, nonalcoholic beers like root beer, mineral water, aerated/carbonated, hot and cold drinks, mocktails, etc. These nonalcoholic beverages perform one or more of the following functions: (i) supply vitamins and nutrient required by the body, (ii) provide energy, (iii) hydrate, (iv) supply of the necessary calories and sugar required by the body, and (iv) some can be used as aperitif or palate stimulant.

Some beverages like soft drink, juice, and apple cider naturally may contain little amounts of alcohol. Distillation of ethanol is often to separate alcoholic drinks into what are commercially called spirit and nonalcoholic drinks. Purification of alcoholic drinks to 0.00% alcohol by volume by distillation is impossible. Furthermore, most drinks with the label nonalcoholic contain 0.5% ABV as it brings more profit than distilling it to 0.05% ABV normally found in products sold by companies specialized in nonalcoholic drinks [24]. It is said by some people that the sign nonalcoholic on drinks is misleading and therefore a threat to recovering alcoholics due to the psychoactive nature of alcohol.

Nonalcoholic beer may be beneficial to other people, but it is not advised to be consumed by a recovering alcoholic. According to a study which was conducted on some Spanish nuns who drank nonalcoholic beer for 45 days and had an increased antioxidant levels in their bloodstream, nonalcoholic beer could have positive impacts on the cardiovascular system. Among the disadvantages are (i) the perception of most people is that nonalcoholic beer does not have the rich flavor of normal alcoholic beer; (ii) most nonalcoholic beer contains a small amount of alcohol, which could result into relapse for recovering alcoholics; (iii) research has made it clear that the smell of any kind of beer may trigger cravings in the alcoholic's mind, and the level of dopamine in the brain may be raised; and (iv) there is a fear of being stigmatized as the only one not drinking alcohol in a social gathering.

10. Conclusions

Conclusively, drinks that contain ethanol are regularly alluded to as mixed refreshment and are either produced by fermentation or distillation. The utilization of which might be related with some underlining hazard factor contingent upon the amount devoured per time. The consistent utilization of alcohol, regardless of the terrible impacts it has on human's life, dependence, alcohol addiction, and withdrawal from which can be dangerous. Alcohol expands the rate of production of free radicals in the body framework and furthermore represses the antioxidant levels along these lines instigating oxidative stress. The unnecessary utilization of alcohol can likewise result in immunodeficiency and immunosuppressant subsequently presenting such individual to various types of infections. The causes of alcoholism range from alcoholism gene, peer pressure, to influence of an alcoholic parent, among others. The three affirmed oral pharmaceuticals for the treatment of alcohol addiction or dependence are disulfiram, naltrexone, and acamprosate. While, nonalcoholic beverages alludes to any drink that contains no alcohol or which contains under 0.5% alcohol by volume. Moderate consumption of alcohol or red wines has been affirmed to be gainful to human well-being especially on

account of the cancer prevention agent properties it gives. Some other positive effects like reduction of diabetes, stroke, and coronary heart disease have been associated with moderate consumption of alcohol particularly among middle-aged and older men and women.

Conflict of interest

The authors hereby declare that there is no conflict of interest.

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Chapter 2

Usage of Kombucha 'Tea Fungus' for Enhancement of Functional Properties of Herbal Beverages

Viduranga Yashasvi Waisundara

Abstract

The following herbal teas were fermented with the Kombucha "tea fungus" for 7 days: *Acacia arabica*, *Aegle marmelos* root bark, *Aerva lanata*, *Asteracantha longifolia*, *Cassia auriculata*, *Hemidesmus indicus*, *Hordeum vulgare*, *Phyllanthus emblica*, *Tinospora cordifolia*. Microbial enumerations of the bacteria and fungi present in the broth and the tea fungal mats were carried out. At the end of the period of fermentation, the pH values ranged from 4.0 to 6.0, while the titratable acidity (TA) ranging from 2.5 to 5.0 g/mL. The TA was within the acceptable limits of consumption for all beverages. The Oxygen radical absorbance capacity (ORAC) assay indicated 5 of the fermented beverages to have statistically significant increases ($P < 0.05$) by the end of the period of fermentation. By day 7, the IC_{50} values of the α-amylase inhibitory activities ranged from 52.5 to 67.2 μg/mL, while the α-glucosidase inhibitory activity values ranged from 95.2 to 196.1 μg/mL by this time point. Overall, an enhancement of the antioxidant and starch hydrolase inhibitory potential of the seven herbal teas was observed as a result of the fermentation by addition of the tea fungus. Thus, this fermentation process could be highlighted as a novel and versatile methodology to obtain functional beverages.

Keywords: antioxidant activity, ORAC, Kombucha, starch hydrolase inhibitory activity, total phenolic content

1. Introduction

Kombucha or "tea fungus" is a fermented beverage which is believed to have its origins in northeast China, and has recently gained rapid popularity among the rest of the world [1]. The beverage is produced through the symbiotic growth of bacteria and osmophilic yeast strains in a thick jelly-like membrane which is cultured in sugared black tea (**Figure 1**) [2]. While the term "Kombucha" is the most commonly used name for the beverage on a commercial basis, it is also known by other names such as Chainii grib, Chainii kvass Champignon de longue vie, Ling zhi, kocha kinoko and red tea fungus [3]. For the production of the beverage, the substrate is incubated with the tea fungal mat statically under aerobic conditions, usually for a minimum of 7 days at 20–28°C [4]. However, to obtain a pleasantly sour beverage with palatability and acceptable sensory properties, the fermentation should terminate when the titratable acidity (TA) reaches 4.0–4.5 g/L—a level which has been confirmed as acceptable by longtime consumers of the Kombucha beverage and is known as the optimal consumable acidity [5].

Figure 1.
Fermented black tea using the Kombucha tea fungal mat after 7 days of fermentation.

There are several types of fermentation processes which take place in the production of the beverage and obtained by-products depending on the various metabolic pathways followed by the microorganisms [6]. Kombucha fermentation is a combination of three such pathways: Alcoholic, lactic, and acetic acid production [6]. These pathways take place primarily because of the presence of several yeasts and bacteria coexisting in the medium, where the fermentation is initiated by osmotolerant microorganisms and ultimately dominated by acid-tolerant species [6].

There are many yeast genus and species in the Kombucha culture, where a broad spectrum has been reported by many researchers including *Zygosaccharomyces*, *Candida*, *Kloeckera*/*Hanseniaspora*, *Torulaspora*, *Pichia*, *Brettanomyces*/*Dekkera*, *Saccharomyces*, *Lachancea*, *Saccharomycodes*, *Schizosaccharomyces*, and *Kluyveromyces* [7–10]. The dominant bacteria of the Kombucha tea culture are acetic acid bacteria, which are aerobic and thus, are able to use alcohol as a substrate to form acetic acid [6]. However, in contrast to yeast, these bacteria require large amounts of oxygen for their growth and activity [6]. Tea provides the necessary nitrogen sources for the bacteria and yeast cultures present in the tea fungal mat [11]. Black tea is the traditional and most dominant substrate used for the Kombucha fermentation, which is independent of its comparatively lower caffeine content (2%) as compared with green tea (5%) [12]. This could be because of the comparatively more acceptable sensory qualities and flavor characteristics generated in black tea as a result of the fermentation process.

Some studies have been able to successfully demonstrate the preparation of fermented beverages through the addition of the tea fungus to various types of plant-based products which are essentially not of *Camellia sinensis* origin [13, 14]. Additionally, Watawana et al. [15] has successfully demonstrated the substitution of sugar with other types of sweetening agents for carrying out the fermentation process of the Kombucha beverage. Considering the therapeutic properties of many of the other herbal teas available in the marketplace, whether their antioxidant potential can be enhanced by natural or artificial means is a reasonable query since value-addition to existing beverages for the purpose of novelty and meeting consumer demands is a matter requiring urgent attention in the functional beverage industry.

In this study, the following plant-based herbal teas were fermented by addition of a locally available tea fungal mat: *Acacia arabica* (AA), *Aegle marmelos* root bark (AM-RB), *Aerva lanata* (Ala), *Asteracantha longifolia* (Alo), *Cassia auriculata* (CA), *Hemidesmus indicus* (HI), *Hordeum vulgare* (HV), *Phyllanthus emblica* (PE), *Tinospora cordifolia* (TC). All these herbs which are commonly consumed in Sri Lanka for health and wellness purposes have almost similar taste and color as black tea. This somewhat reassures that the fermented product does not essentially result in any adverse sensory properties which may discourage consumer acceptability.

The selection of herbs was also based with the intention of widening the application of the tea fungus to other types of herbal teas which maybe more readily available depending on the vegetation of various regions and countries. Jayawardena et al. [16] has investigated the antioxidant and starch hydrolase inhibitory potential of these herbs, further justifying their selection as a fermentation medium for preparation of the novel beverages. Identification of the dominant bacteria and yeast species were carried out as well as the changes to the overall population of bacteria and yeast in the broth and pellicle. Other than pH and TA, the enhancement of the antioxidant potential was evaluated. Another aspect of this study was the investigation of the starch hydrolase inhibitory activities, namely α-amylase and α-glucosidase enzyme activities, of the fermented beverages. Starch hydrolase inhibitors retard the absorption of glucose by curbing the action of α-amylase and α-glucosidase [17]. Compounds which are able to impede these enzymes and thereby, delay starch digestion, resulting in a reduction in the rate of glucose absorption, which in turn blunts the postprandial plasma glucose increase in diabetic patients. The enhancement of the starch hydrolase inhibitory potential of the herbal teas through addition of the tea fungal mat was also investigated in this study.

2. Materials and methods

Acetobacter aceti was found to be the dominant bacterial strain present in the tea fungal mat which was used for the study. The dominant yeast components were identified as *Zygosaccharomyces bailii* and *Brettanomyces claussenii*. These aspects were confirmed through DNA sequencing as per the method by Marsh et al. [14]. Dried powders of the plants were obtained from the Ayurveda Medicinal Hall in Kandy, Sri Lanka during the months of June–July 2014.

2.1 Preparation of the fermented beverages and determination of pH, TA and overall population of bacteria and yeast in the broth and pellicle

One gram each of the plant powders were added to 100 mL of boiling water and infused for 5 min followed by filtration through a sterile sieve. Sucrose (10%) was dissolved in each beverage and the preparation was left to cool to room temperature at 24 ± 3°C. The cooled teas were aseptically inoculated with 10 mL of the freshly grown tea fungal broth which was originated from black tea for 7 days at 24 ± 3°C. Sampling was performed on a daily basis in order to avoid contamination. Broths of the fermented beverages were centrifuged at 7240 g for 10 min prior to the assays to remove any particulate matter present as a result of microbial action and coalescence of proteins, which might interfere with the measurements. An electronic pH meter (Orion model 290A) was used to measure the pH of the broths, while the TA was measured according to the method by Chen and Liu [12] using acid-based titration. Fermented broth (10 mL) was titrated with 0.1 M NaOH and the end-point was determined by measuring the pH using the electronic pH meter (Orion model 290A), where pH = 7.0 was taken as the end-point. The final TA of the fermented broths was expressed in g/mL.

Changes to the overall population of bacteria and yeast in the fermented broth and pellicle was determined according to the method by Chen and Liu [12]. Glucose-yeast extract-calcium carbonate agar (GYCA) and potato dextrose agar (PDA) media, were used for the growth of bacteria and yeast, respectively. As per the method by Chen and Liu [12], the GYCA medium was composed of 30 g of glucose, 5 g of yeast extract, 3 g of peptone, 10 g of calcium carbonate, 30 mL

of 95% ethanol, 20 g of agar, and distilled water added to make a final volume of 1 L. Aliquots of 1 mL were taken from both the broth and the tea fungal mat, while the upper pellicle portion was filtered with a sterile cheesecloth before sampling to remove the cellulose fibers. Both bacterial and yeast counts were expressed as colony-forming units per mL (cfu/mL).

2.2 Determination of the total phenolic content (TPC) and antioxidant activity

The method by Huang et al. [18] was used for determining the TPC. The results were expressed as milligrams of gallic acid equivalents (GAE) per milliliters (mg GAE/mL), using a gallic acid standard curve. The assay was carried out in 96-well format where the following constituent volumes were added in this particular order to a single well to add up to a total volume of 200 μL per well: Sample / blank (deionized water) / gallic acid standard—20 μL, Folin-Ciocalteu reagent—100 μL, Na_2CO_3 (30 g/L)—80 μL. The microplate was incubated at room temperature for 15 min following which the absorbance was read at 540 nm using a Thermo Scientific Multiskan GO Microplate Reader. The TPC value was back-calculated using the gallic acid standard curve.

The Oxygen Radical Absorbance Capacity (ORAC) assay was carried out according to the methodology by Prior et al. [19]. The final values for this assay were expressed as micromoles of trolox equivalents (TE) per milliliters (μmol TE/mL) using a trolox standard curve. Trolox is a vitamin E analogue and is ideal for the calculation of the ORAC value. This assay was carried out in 96-well format as well [19]. The following components were added to a single well in this particular order, adding up to a total volume of 200 μL per well: (1) Blank (phosphate buffered saline)/ trolox standard/sample—20 μL, (2) fluorescein working solution—160 μL, (3) 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)—20 μL. AAPH was added in last since it is a radical initiator and for the reaction kinetics to occur in all wells at the same time point. The reaction kinetics were monitored for two hours as a fluorescence decay [485 nm (ex)/525 nm (em)], following which the area under the curve was used to calculate the ORAC value compared with those of the trolox standards.

As for the di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) radical scavenging activity assay, 100 μL of each of the broths of the fermented beverages were mixed with 1 mL of 0.1 mM DPPH in ethanol along with 450 L of 50 mM Tris-HCl buffer at pH 7.4. The solution was incubated at room temperature for 30 min and reduction of DPPH radicals was measured by reading the absorbance of the resulting solution at 517 nm. The antioxidant activity was calculated as % DPPH radical scavenging activity. Scavenging ability of superoxide radical (O_2^-) was assessed by the method described by Lee et al. [20], and was carried out in 96-well format using the Thermo Scientific Multiskan GO Microplate Reader similar to the TPC and ORAC assays.

2.3 Determination of the α-amylase and α-glucosidase inhibitory activities

The α-amylase inhibitory activity was evaluated according to the method by Liu et al., where acarbose was used as the positive control [17]. The reduction of turbidity at 660 nm was monitored over time using the Thermo Scientific Multiskan GO Microplate Reader, and the area under the curve was used to calculate the overall inhibitory activity. The α-glucosidase inhibitory activity was carried out according to the method by Koh et al., and the same positive control was used in this instance as well [21]. A reaction substrate consisting of 4-nitrophenyl R-D-glucopyranoside (PNPG), (30 mM) and α-glucosidase (16.65 mg/mL) were prepared in phosphate

buffer saline (PBS). A volume of 340 μL of inhibitor solutions of different concentrations was pipetted into separate reaction vials. Then, 20 μL of the α-glucosidase solution was added into each vial, and incubated at 37°C for 10 min. The PNPG solution (40 μL) was added to initiate the digestion. After 15 min, 200 μL of 1 M Na_2CO_3 solution was added to terminate the reaction. Aliquots of 300 μL from the reacted solutions were withdrawn and added each well of a 96-well microplate. Absorbance at 400 nm was read using the Thermo Scientific Multiskan GO Microplate Reader.

For both assays, experiments were performed in triplicate, and a curve of percentage inhibition against inhibitor concentration was plotted with the averaged values to back-calculate to the enzyme inhibitory values in the samples. Although for both assays, acarbose equivalence could also be used to express the final enzyme inhibitory data, in this instance, for ease of reference and comparison, the results were expressed as IC_{50} (mg/mL).

2.4 Statistical analysis

For the statistical evaluations, IBM SPSS Statistics version 21.0 released in 2012 (IBM Corp., Armonk, NY, USA) for Windows was used. Three or more independent analyses were utilized to calculate and express the results as mean ± standard error mean (SEM). *P* values of >0.05 were considered to be significant.

3. Results and discussion

As per **Figure 2**, all the beverages had a statistically significant decrease ($P < 0.05$) in the pH values by the end of the fermentation process on day 7. Out of the nine fermented beverages, HI and TC had statistically significant decreases ($P < 0.05$) in the pH from day 1 itself, whereas changes took place slightly later in the rest of the beverages. The decrease in pH of all the Kombucha samples as a whole would have been due to the increased concentration of organic acids (typically in the form of acetic acid) produced during the fermentation process. As per **Figure 1**, all unfermented teas had an initial pH value of between 6.0 and 8.0 prior to fermentation, while the pH values were between 4.0 and 6.0 on day 7 at the end of the fermentation period.

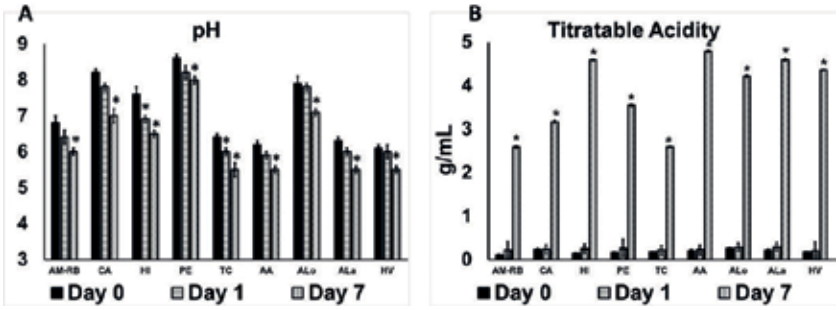

Figure 2.
*The (A) pH and (B) titratable acidity (TA) of the beverages prior to addition of the tea fungal mat (day 0), followed by day 1 and day 7 of fermentation. Values are expressed as mean ± SEM. * P < 0.05 versus the value of each tea at day 0. Abbreviations: Acacia arabica (AA), Aegle marmelos root bark (AM-RB), Aerva lanata (Ala), Asteracantha longifolia (Alo), Cassia auriculata (CA), Hemidesmus indicus (HI), Hordeum vulgare (HV), Phyllanthus emblica (PE), Tinospora cordifolia (TC).*

As per **Figure 2**, the TA of all the unfermented beverages had initial values ranging from 0.1 to 0.5 g/mL. Once the fermentation was complete by day 7, the TA values ranged between 2.5 and 5.0 g/mL, where all beverages had statistically significant increases in this particular parameter ($P < 0.05$). Given that the optimum consumable acidity level is 4.0–4.5 g/L for Kombucha beverages, only AA and HI did not have TA levels falling within this range by the end of the period of fermentation. Nevertheless, on a comparative basis, according to the TA reference levels as indicated by Reiss [5], it may be assumed that all of the Kombucha beverages were acceptable for consumption in terms of acidity.

The TPC and ORAC values are shown in **Figure 3**. AM-RB had the highest TPC prior to fermentation with HV being the lowest. By day 7, statistically significant increases ($P < 0.05$) in the TPC were observed in AM-RB, CA, HI and TC. Correlating with the TPC values, AM-RB had the highest ORAC values with HV being the lowest. The DPPH EC_{50} values appear to have complemented the ORAC values with the exceptions of AM-RB, TC, AA and ALa, where their values were observed to have statistically significant increases ($P < 0.05$) by day 7 (**Figure 3**).

As for the antioxidant assay of superoxide scavenging activity, the trends of increase or decrease whether it be statistically significant or not, were not as clear as the ORAC and DPPH EC_{50} values (**Figure 4**). A better correlation between the TPC and the ORAC values rather than the DPPH EC_{50} and superoxide scavenging values of all beverages on all days of analysis ($R^2 = 0.985$ for ORAC versus $R^2 = 0.745$ DPPH EC_{50} and $R^2 = 0.632$ for superoxide scavenging potential). This showed that the phenolic compounds present in the fermented beverages were better scavengers of the peroxyl radical which is generated through the AAPH.

The starch hydrolase inhibitory activities of α-amylase and α-glucosidase enzymes are shown in **Figure 5**. On the first day of fermentation, only CE, HI, PE and AA had statistically significant increases ($P < 0.05$) in the α-amylase inhibitory activities. However, by day 7 at the end of the fermentation period, all the beverages had statistically significant increases ($P < 0.05$) as compared with day 0. As for the α-glucosidase inhibitory activities, AM-RB, CA, HI, PE, AA, ALa and HV had statistically significant increases ($P < 0.05$) during both day 1 and 7 as compared with day 0. The fermentation process was able to enhance the

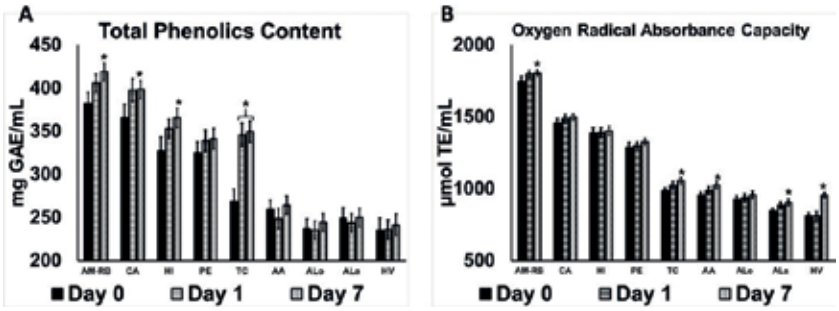

Figure 3.
*The (A) Total phenolic content (TPC) and (B) ORAC values of the herbs prior to addition of the tea fungal mat (day 0), followed by day 1 and day 7 of fermentation. Values are expressed as mean ± SEM. * $P < 0.05$ versus the value of each tea at day 0. Abbreviations: Acacia arabica (AA), Aegle marmelos root bark (AM-RB), Aerva lanata (Ala), Asteracantha longifolia (Alo), Cassia auriculata (CA), Hemidesmus indicus (HI), Hordeum vulgare (HV), Phyllanthus emblica (PE), Tinospora cordifolia (TC).*

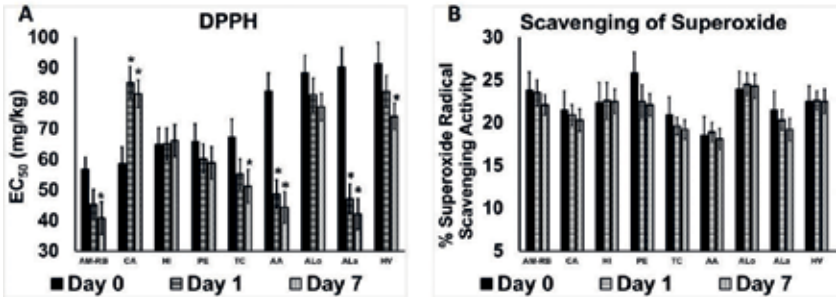

Figure 4.
*The (A) DPPH and (B) superoxide radical scavenging values of the herbs prior to addition of the tea fungal mat (day 0), followed by day 1 and day 7 of fermentation. Values are expressed as mean ± SEM. * P < 0.05 versus the value of each tea at day 0. Abbreviations: Acacia arabica (AA), Aegle marmelos root bark (AM-RB), Aerva lanata (Ala), Asteracantha longifolia (Alo), Cassia auriculata (CA), Hemidesmus indicus (HI), Hordeum vulgare (HV), Phyllanthus emblica (PE), Tinospora cordifolia (TC).*

α-amylase inhibitory activity better than the α-glucosidase inhibitory activity. This is an important aspect, given that α-amylase is required for the subsequent reactions of α-glucosidase, and therefore, curbing the reactivity of α-amylase is important from the perspective of reducing the release of simple sugars into physiological system [22].

Changes to the composition of the overall population of bacteria and yeast present in the broth and pellicle on days 1 and 7 are shown in **Tables 1** and **2**, respectively. As per the measurements from day 0, the inoculum of 10 mL which was added to initiate the fermentation process contained various numbers of bacteria and yeast. However, overall, an increase in the bacteria and yeast population in all of the fermented beverages was observed by the end of the period of fermentation. The increase in these numbers was indicative of the microbes' viability and successful livelihood in the beverages, thus, confirming their ability of thrive in the environments in similar fashion to black tea. An aspect which remains to be investigated concerning this parameter is whether some of the microbes have entered a viable but nonculturable (VBNC) state. Since the origins of the bacteria and yeast are from black tea itself, it is possible that some of the microbes have entered a state of low metabolic activity, and thus were not accounted for during the measurement of this parameter.

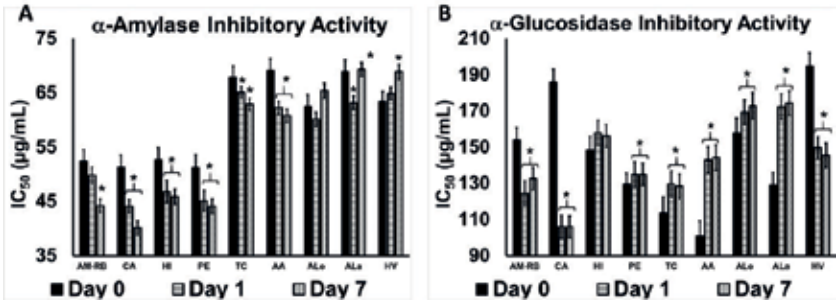

Figure 5.
*α-amylase and α-glucosidase inhibitory activity values of the herbs prior to addition of the tea fungal mat (day 0), followed by day 1 and day 7 of fermentation. Values are expressed as mean ± SEM. * P < 0.05 versus the value of each tea at day 0. Abbreviations: Acacia arabica (AA), Aegle marmelos root bark (AM-RB), Aerva lanata (Ala), Asteracantha longifolia (Alo), Cassia auriculata (CA), Hemidesmus indicus (HI), Hordeum vulgare (HV), Phyllanthus emblica (PE), Tinospora cordifolia (TC).*

Herb	Microbes	Days		
		0 (cfu/mL)	1 (cfu/mL)	7 (cfu/mL)
AA	Bacteria	$2.9 \pm 0.1 \times 10^6$	$8.6 \pm 0.2 \times 10^6$	$3.9 \pm 0.3 \times 10^9$
	Yeast	$1.5 \pm 0.2 \times 10^6$	$8.9 \pm 0.1 \times 10^6$	$9.2 \pm 0.1 \times 10^9$
AM-RB	Bacteria	$3.9 \pm 0.1 \times 10^6$	$6.5 \pm 0.3 \times 10^6$	$9.1 \pm 0.1 \times 10^{10}$
	Yeast	$6.9 \pm 0.2 \times 10^6$	$6.6 \pm 0.2 \times 10^7$	$9.5 \pm 0.1 \times 10^{10}$
ALa	Bacteria	$3.5 \pm 0.1 \times 10^6$	$6.6 \pm 0.2 \times 10^7$	$7.5 \pm 0.2 \times 10^9$
	Yeast	$5.3 \pm 0.1 \times 10^6$	$9.3 \pm 0.2 \times 10^5$	$9.6 \pm 0.1 \times 10^{10}$
ALo	Bacteria	$3.4 \pm 0.2 \times 10^6$	$6.6 \pm 0.2 \times 10^6$	$9.8 \pm 0.1 \times 10^{10}$
	Yeast	$5.1 \pm 0.2 \times 10^6$	$5.5 \pm 0.1 \times 10^7$	$1.8 \pm 0.1 \times 10^{10}$
CA	Bacteria	$1.9 \pm 0.2 \times 10^6$	$2.5 \pm 0.1 \times 10^6$	$3.6 \pm 0.1 \times 10^{10}$
	Yeast	$1.2 \pm 0.2 \times 10^6$	$8.8 \pm 0.2 \times 10^6$	$9.2 \pm 0.1 \times 10^{10}$
HI	Bacteria	$3.6 \pm 0.2 \times 10^6$	$5.9 \pm 0.1 \times 10^6$	$3.9 \pm 0.1 \times 10^{10}$
	Yeast	$5.3 \pm 0.1 \times 10^6$	$6.5 \pm 0.2 \times 10^7$	$9.0 \pm 0.1 \times 10^{10}$
HV	Bacteria	$4.1 \pm 0.1 \times 10^6$	$3.5 \pm 0.3 \times 10^6$	$4.8 \pm 0.1 \times 10^{10}$
	Yeast	$2.5 \pm 0.1 \times 10^6$	$4.4 \pm 0.3 \times 10^5$	$8.0 \pm 0.2 \times 10^{10}$
PE	Bacteria	$1.2 \pm 0.2 \times 10^6$	$3.9 \pm 0.2 \times 10^6$	$7.1 \pm 0.1 \times 10^9$
	Yeast	$3.2 \pm 0.1 \times 10^6$	$5.0 \pm 0.1 \times 10^7$	$8.0 \pm 0.2 \times 10^9$
TC	Bacteria	$1.9 \pm 0.2 \times 10^6$	$3.2 \pm 0.1 \times 10^6$	$2.3 \pm 0.2 \times 10^9$
	Yeast	$4.1 \pm 0.1 \times 10^6$	$8.3 \pm 0.2 \times 10^6$	$1.9 \pm 0.1 \times 10^{10}$

Abbreviations: AA, Acacia arabica; AM-RB, Aegle marmelos root bark; Ala, Aerva lanata; Alo, Asteracantha longifolia; CA, Cassia auriculata; HI, Hemidesmus indicus; HV, Hordeum vulgare; PE, Phyllanthus emblica; TC, Tinospora cordifolia.

Table 1.
Changes to the composition of the overall population of bacteria and yeast present in the broth prior to fermentation (day 0) as well as on day 1 and day 7.

The hypothesis for the increase in the TPC was explained by Blanc [23], where phytases liberated by bacteria and yeast in the tea fungus consortium were identified as capable of liberating polyphenol compounds from the cellulosic backbone of the fermentation medium. This metabolic reaction would have resulted in an increase in the polyphenols in the soluble fraction of the fermented beverages produced in this study. Phytases liberated by bacteria and yeast during Kombucha fermentation are also able of causing degradation of complex polyphenols to smaller molecules which would also result in the increase of TPC. The therapeutic effect of the Kombucha fermentation process has been associated with the increased presence of polyphenols, compounds produced during the fermentation period, and synergistic action between different compounds which are liberated at various stages of the fermentation process [24–26].

The opportunities and challenges for the food and beverage industry in the area of evidence-based functional foods with a low glycemic index which are able to curb the starch digestion rates are on the rise, given the increasing incidence of diabetes throughout the world [27]. Given the noteworthy starch hydrolase inhibitory activities of the fermented herbal beverages generated in this study, their importance in

Herb	Microbes	Days	
		1 (cfu/mL)	7 (cfu/mL)
AA	Bacteria	$3.7 \pm 0.2 \times 10^6$	$2.2 \pm 0.1 \times 10^8$
	Yeast	$1.1 \pm 0.2 \times 10^5$	$1.6 \pm 0.2 \times 10^7$
AM-RB	Bacteria	$1.1 \pm 0.1 \times 10^6$	$1.5 \pm 0.1 \times 10^8$
	Yeast	$1.5 \pm 0.2 \times 10^5$	$5.5 \pm 0.2 \times 10^7$
ALa	Bacteria	$6.6 \pm 0.2 \times 10^6$	$7.5 \pm 0.1 \times 10^8$
	Yeast	$1.5 \pm 0.1 \times 10^5$	$9.2 \pm 0.1 \times 10^7$
ALo	Bacteria	$2.1 \pm 0.2 \times 10^6$	$9.5 \pm 0.1 \times 10^8$
	Yeast	$1.6 \pm 0.2 \times 10^5$	$1.9 \pm 0.1 \times 10^7$
CA	Bacteria	$2.8 \pm 0.1 \times 10^6$	$2.8 \pm 0.1 \times 10^8$
	Yeast	$5.5 \pm 0.1 \times 10^5$	$4.8 \pm 0.2 \times 10^7$
HI	Bacteria	$3.9 \pm 0.1 \times 10^6$	$3.9 \pm 0.1 \times 10^8$
	Yeast	$4.9 \pm 0.1 \times 10^5$	$5.9 \pm 0.3 \times 10^7$
HV	Bacteria	$4.4 \pm 0.2 \times 10^6$	$7.5 \pm 0.1 \times 10^8$
	Yeast	$4.7 \pm 0.1 \times 10^5$	$5.5 \pm 0.1 \times 10^7$
PE	Bacteria	$3.5 \pm 0.1 \times 10^6$	$6.6 \pm 0.2 \times 10^8$
	Yeast	$2.2 \pm 0.1 \times 10^5$	$2.9 \pm 0.1 \times 10^7$
TC	Bacteria	$3.1 \pm 0.1 \times 10^6$	$2.5 \pm 0.1 \times 10^8$
	Yeast	$6.6 \pm 0.1 \times 10^5$	$1.9 \pm 0.2 \times 10^7$

Abbreviations: AA, Acacia arabica; AM-RB, Aegle marmelos root bark; Ala, Aerva lanata; Alo, Asteracantha longifolia; CA, Cassia auriculata; HI, Hemidesmus indicus; HV, Hordeum vulgare; PE, Phyllanthus emblica; TC, Tinospora cordifolia.

Table 2.
Changes to the composition of the overall population of bacteria and yeast present in the pellicle on day 1 and day 7.

support of preventing metabolic diseases such as diabetes could be highlighted in this aspect. Recently, there have been many warnings on the side effects of anti-diabetic drugs such as Rosiglitazone and Pioglitazone which highlight the urgent need of alternative and safer means of blood glucose control—a prospect which could be ideally achieved through functional foods which contain bioactive ingredients with the ability to regulate blood glucose concentration toward the normal range [28].

Last but not least, the selection of a particular fermentation period for this study requires justification. The beverages and their analytical parameters were monitored for 7 days. This was the minimum duration by which the fermented beverages could be consumed without the presence of metabolic artifacts resulting from prolonged fermentation according to literature [25, 29, 30]. In particular, according to the study by Amarasinghe et al. [30] where the study was conducted for monitoring the fermentation for a period of 8 weeks, the Kombucha samples displayed a decrease in the antioxidant activity during the 2 months of fermentation. This was suggestive that suggestive the functional properties of the beverage had decreased. It was also implied in this study that it is possible through prolonged fermentation to result in the accumulation of organic acids, which might reach harmful levels for direct consumption [30].

4. Conclusions

Given the ease of preparation of the beverages which were investigated in this study, as well as the economic viability, the products could be promoted as functional beverages which could be consumed as means of supportive therapy. In particular, they could be used for the prevention and containment of disease conditions in association with their demonstrated antioxidant and starch hydrolase inhibitory properties. Although the therapeutic mechanisms of action of these beverages in the human physiology is yet to be elucidated. However, this study serves as a platform for the identification and promotion of some novel beverages as functional food which can be easily prepared in households using edible plants. Nevertheless, having mentioned as such, during the selection of plant material for fermentation with the Kombucha tea fungal mats, care needs to be taken to identify plant-based material which carry similar sensory perceptions as black tea, thus, reducing the chances of the fermented products possessing adverse sensory perceptions.

During this study, the sensory evaluations of the novel fermented beverages was not carried out. This is one aspect which needs to be explored in future studies. It is only hypothesized in this instance that the organoleptic properties would be acceptable, since the teas are similar in physical properties to *Camellia sinensis*-based tea. However, for a proper verification, sensory evaluation needs to be carried out.

The microbial interactions taking place during the fermentation needs to be investigated further as well. For instance, as mentioned previously, whether the microbes undergo a state of VBNC needs to be elucidated. Additionally, whether microorganisms which have probiotic effects such as *Lactobacillus* spp., and *Bifidobacterium* are able to thrive in the fermented beverage and thus be incorporated, is an aspect worth exploring as well. Should this be successful, the consumer value of the beverages would increase and have a superior marketability due to its combined functional properties of comprising of phenolic compounds as well as probiotics.

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Conflict of interest

The author has no conflicts of interest to declare, financial or otherwise.

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Chapter 3

The Role of UV-Visible Spectroscopy for Phenolic Compounds Quantification in Winemaking

Jose Luis Aleixandre-Tudo and Wessel du Toit

Abstract

Phenolic compounds are bioactive substances present in a large number of food products including wine. The importance of these compounds in wine is due to their large effect on the organoleptic attributes of wine. Phenolic compounds play a crucial role in the colour as well as mouthfeel properties of wines. UV-visible spectroscopy appears as a suitable technique for the evaluation of phenolic compounds' properties and content. The ability of the phenolic ring to absorb UV light and the fact that some of the phenolic substances are coloured compounds, i.e. show absorption features in the visible region, make UV-visible spectroscopy a suitable technique to investigate and quantify grape and wine phenolic compounds. A number of analytical techniques are currently used for phenolic quantification. These include both simpler approaches (spectrophotometric determinations) as well as more complex methodologies such liquid chromatography analysis. Moreover, a number of spectroscopy applications have also been recently reported and are becoming popular within the wine industry. This chapter reviews information on the UV-visible spectral properties of phenolic compounds, changes occurring during wine ageing and also discusses the current UV-visible based analytical techniques used for the quantification of phenolic compounds in grapes and wine.

Keywords: UV-visible, spectrophotometry, phenolic compounds, anthocyanins, tannins, liquid chromatography, spectroscopy, chemometrics, fluorescence

1. Introduction

Phenolic compounds are bioactive molecules that are involved in some of the most relevant wine organoleptic attributes. Phenolic substances have been reported as being responsible for wine colour, mouthfeel perception and flavour. The appropriate management of the phenolic accumulation in the berry, extraction during the skin contact phase as well as the evolution during ageing in barrels or bottles will ensure a desired phenolic content and composition that will lead to a good quality wine [1]. Furthermore, the ability of phenolic molecules to act as antioxidant has placed this group of compounds in the spotlight of a considerable amount of research. Phenolic compounds have been reported as effective antioxidants and their preventive role against inflammatory, neurodegenerative, cardiovascular

diseases or even against cancer has been widely acknowledged [2]. The quantification of phenolic compounds is thus of high importance and UV-visible spectroscopy has proven to be one of the most suitable and reliable techniques to quantify these substances during the winemaking process.

The accumulation of the amino acid phenylalanine is the first step towards the biosynthesis of phenolic compounds. Phenolic substances or polyphenols are thus secondary metabolites that contain at least one aromatic ring and one or several hydroxyl groups. Two main families of phenolic compounds are generally classified as the non-flavonoids and the flavonoids. Phenolic acids, including hydroxycinnamic and hydroxybenzoic acids and stilbens are part of the structurally less complex non-flavonoid group (**Figure 1**). Flavonoids share a common C3-C6-C3 structure and contain flavonols, anthocyanins and flavanols, with the latter also known as proanthocyanidins or more widely as tannins [3]. The biosynthesis and accumulation of these key substances is due to a number of plant biological functions which include growth, plant reproduction and plant protection roles against environmental signals as well as biotic and abiotic stresses [4].

Phenolic compounds are released from the solid parts of the berries into the must during the winemaking process. The contact period refers to the period of time that the must is in contact with the skins and seeds and generally coincides with the alcoholic fermentation. The presence or absence of the solid parts during the winemaking process will determine the phenolic content and composition. In white winemaking the skin contact period is limited to a minimum and the levels of phenolic compounds found in wines are thus lower than in red wines (where the fermentation takes place in the presence of skins and seeds). Due to its location in the flesh, hydroxycinnamic acids are therefore the main phenolic compounds found in white wines. On the contrary, red wines contain high levels of tannins, anthocyanins and flavonol compounds that are extracted from the solid parts of the berries during the aforementioned skin contact phase [5].

Among the subclasses of phenolic compounds found in grapes, two of the subfamilies are mostly of importance to wine production. Anthocyanins are coloured compounds responsible for the red wine colour attributes. The state of the anthocyanins and the wine medium conditions have a major impact on the final wine colour. Anthocyanins are found in red grapes and wines in five mono-glucoside forms. The 3-glucoside forms of delphinidin, cyanidin, petunidin, peonidin and malvidin are present in *Vitis vinifera* cultivars (**Figure 2**). Monomeric anthocyanins are highly reactive substances involved in a large number of reactions and interactions. Simple anthocyanins are acylated with a number of grape components such as acetic acid, *p*-coumaric or caffeic acid, they are also able to combine with themselves through intra- and intermolecular copigmentation interactions [6]. During the winemaking and ageing processes further reactions to form pyranoanthocyanins have also been documented, in combination with several associations with tannins, some of them through acetaldehyde mediated reactions. Anthocyanin interactions and reactions lead to a number of complex pigments with increased

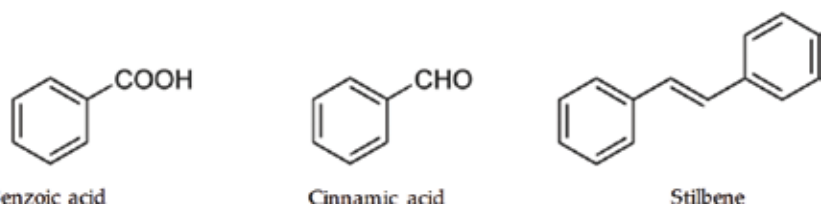

Figure 1.
Chemical structure of the non-flavonoid group of phenolic compounds found in grapes and wines.

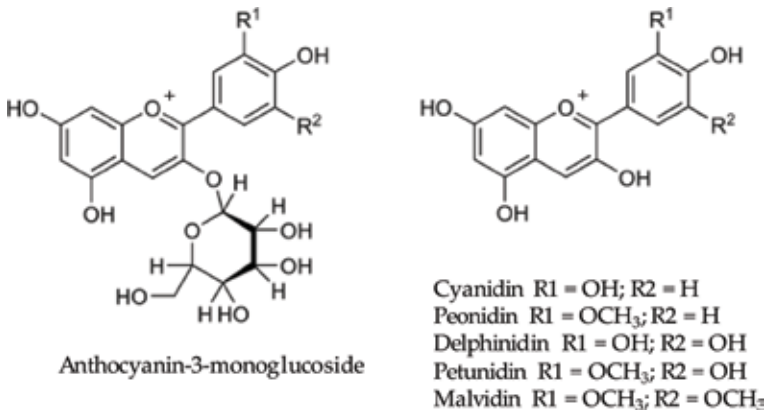

Figure 2.
Chemical structure of the main anthocyanins found in grapes and wines.

stability during wine ageing. These combinations also entail a modification of the anthocyanin coloration, phenomena that gives rise to the large variety of red and brown based colours found in red wines [7]. Additionally, tannin-anthocyanin interactions give rise to a decrease in the ability of tannins to elicit astringency [8].

Proanthocyanidins or tannins are the most abundant class of phenolic compounds. Tannins are polymeric compounds of varying size and structure, containing a combination of five flavanol monomers. The polymerisation of catechin, epicatechin, gallocatechin, epigallocatechin and catechin gallate subunits gives rise to larger and more polymerised tannin compounds (**Figure 3**) with varying ability to elicit astringency and bitterness [3, 9]. The reactivity of the hydroxyl groups towards salivary proteins creates a macromolecular complex that precipitates from solution and leads to a puckering and drying sensation, also known as astringency. Small molecular weight tannins are initially bitter and they became more astringent as the molecular size increases [9]. Young wines, initially more astringent, contain tannins that have been polymerising and have therefore and increased ability to react with salivary proteins. During the ageing process several phenomena explain why the wines become softer (less astringent). When a certain molecular size is reached the tannin molecule may become insoluble, thus precipitating from solution and lowering the tannin content of the wines. Moreover, as molecules grow in size, its conformation might hinder the tannin protein interactions which will also lead to decreased astringency intensity. It is also possible that large tannin molecules cleavages give rise to smaller and less astringent tannins. Finally, the tannin-anthocyanin combinations that take place during the ageing process may also be involved in the decrease of the astringency intensity experienced in older wines [1, 10].

The use of UV and visible light for the quantification of chemical compounds is a widely used technique [1]. Due to their biochemical and molecular properties, phenolic compounds are highly suitable to be quantified with UV-visible light. The ability of the phenolic ring to absorb UV light is exploited to quantify these compounds [11]. In addition to this, visible light can also provide valuable information due to the coloured nature of some of the phenolic compounds (e.g. red anthocyanins or yellow flavonols). The UV-visible spectra of a wine is thus attributed to the electronic transitions occurring within the hydroxyl groups of the phenolic molecules, with different transitions corresponding to the different phenolic subclasses [12]. A number of UV-visible applications have been exploited to quantify phenolic compounds. Among these the use of UV-visible spectrophotometry to estimate the content of phenolic compounds stands out as the most widely used approach. A number of methods have been optimised for the different phenolic subclasses, making nowadays the efficient estimation of

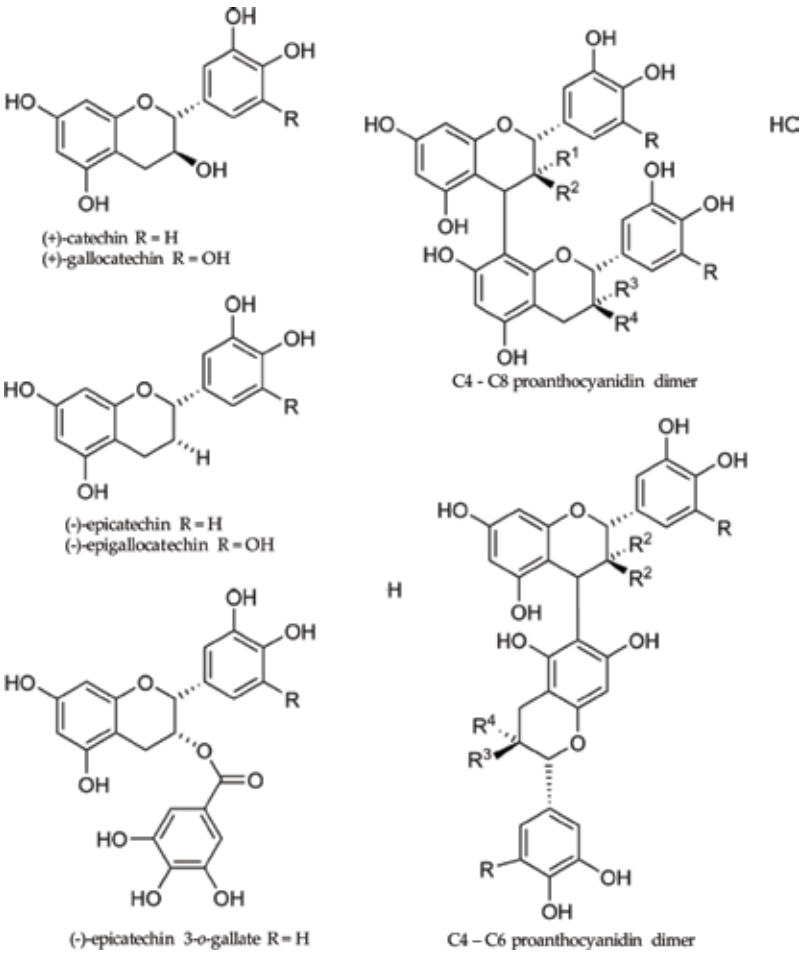

Figure 3.
Chemical structure of flavan-3-ol compounds found in grapes and wines.

phenolic content using a simple UV-visible spectrophotometer possible [1]. However, UV-visible spectroscopy is also used in more advanced separation techniques, such as liquid chromatography, that allows for the quantification of individual phenolic compounds [13]. The quantification of phenolic compounds is thus achieved through the UV-visible signal given by the individually separated phenolic compounds. On the other hand, fluorescence spectroscopy also makes use of the UV-visible spectral features of the excited substances. After the excitation process a coloured fluorophore is quantified based on its absorption intensity projected in the visible region [14]. Finally, UV-visible spectroscopy combined with chemometrics is also included in the techniques used for phenolic compounds' quantification [15]. In this case the spectral properties are used to predict the phenolic content of a given grape phenolic extract or wine [16]. This approach makes use of partial least squares regression analysis to correlate spectral information with reference data (phenolic levels). If successfully performed, a validated calibration can provide accurate predictions of phenolic content by only measuring the UV-visible spectral properties of wines.

This manuscript, in its different sections, reports therefore the current status of the different analytical techniques available for the quantification of phenolic content in grapes and wines. Moreover, the UV-visible spectral features observed in wines during the winemaking process, from the early stages of fermentation and through the ageing process are also reported and discussed.

2. UV-visible features of wines during winemaking and ageing

Among other analytical techniques, UV-visible spectroscopy appears to be suitable for the quantification of phenolic compounds. This is due to two main reasons. First of all, phenolic substances have the ability to strongly absorb UV light [11] and secondly, certain compounds due to the coloured nature can lead to absorption features in the visible range [17]. Polyphenols are biological compounds containing π conjugated systems with hydroxyl-phenolic groups. The π type molecular orbitals electronic transitions provide the UV-visible spectrum of this group of compounds. UV-visible spectroscopy is used in winemaking to quantify different sub-groups within the phenolic family [18]. The most common procedures for phenolic analysis are reported to quantify anthocyanins, phenolic acids (including hydroxycinnamates and hydroxybenzoates), stilbenes, flavonols and flavanols or tannins.

The main absorption feature of the flavanol monomers is a strong absorption band around 280 nm (**Figure 4c**). These colourless compounds do not show absorption features in the visible region of the electromagnetic spectrum. The flavanol monomers may contain a galloyl molecule attached to the flavan-3-ol structure. A galloylated flavanol has been reported to have higher absorption intensity, when compared to its non-galloylated form, it also shows a shoulder at 310 nm, characteristic of the galloyl group (see gallic acid as example in **Figure 4d**) [11]. For flavanol polymers or tannins the absorption features remain the same despite the degree of polymerisation (number of monomers) of the proanthocyanidin structure with a predominant absorption band at 280 nm.

Anthocyanin compounds co-exist under different forms and its colour intensity and tonality depends on the proportion of the different molecular structures

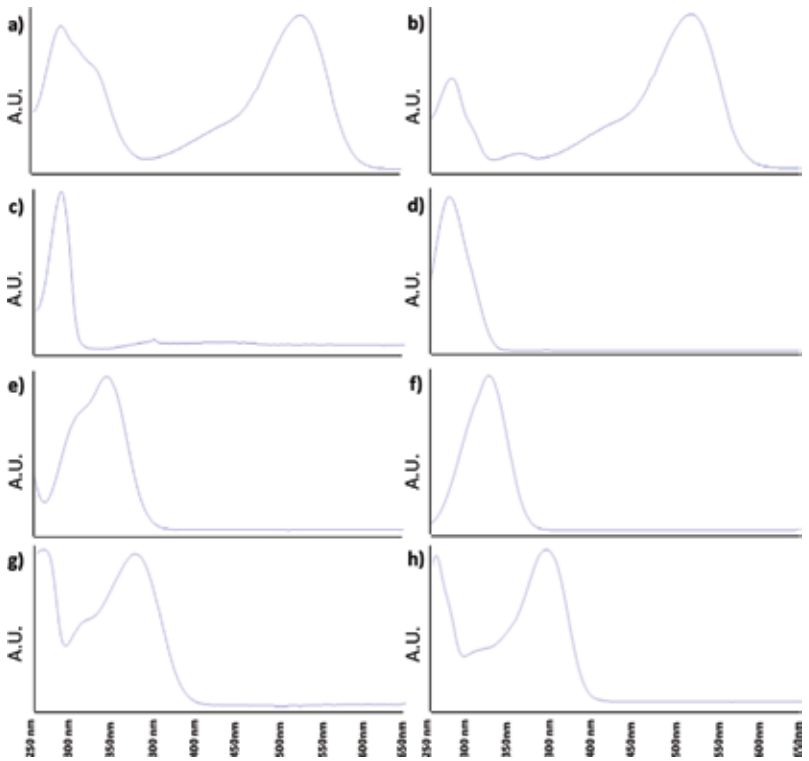

Figure 4.
UV-vis spectral properties of individual phenolic compounds. (a) Malvidin-3-glucoside, (b) malvidin-3-p-coumarylglucoside, (c) catechin, (d) gallic acid, (e) caftaric acid, (f) coutaric acid, (g) rutin, (h) quercetin.

present at the time of evaluation. The main absorption features of this phenolic subfamily are given by an intense absorption band at 280 nm, common to all phenolic substances, and by a characteristic absorption intensity around 520 nm characteristic of red colouring substances (**Figure 4a**). In addition, the anthocyanins are found in grapes and wines acylated with a number of other wine components, including some phenolic acids such as caffeic or *p*-coumaric acids [3]. In this case the anthocyanin molecule will also show a characteristic broad band around 320 nm (see malvidin-3-*p*-coumarylglucoside in **Figure 4b**). Anthocyanins are highly reactive phenolic compounds strongly influenced by the pH conditions and by the presence of SO_2 [19]. Lower pH values increase the proportion of anthocyanins present in the red flavylium form, leading to increased colour intensity, through an hyperchromic effect in the visible region. The opposite behaviour is thus observed if the wine's pH increases to higher values, leading to a decrease of the absorption intensity at 520 nm (hypochromic effect). On the other hand, the ability of anthocyanins to exist in its red forms is highly dependent on the SO_2 content. Sulphur dioxide has the ability to interact and combine with the anthocyanin molecule in position 4 of the central phenolic ring, causing the decolouration of the chromophore, leading to a colourless flavilium sulphonate [6]. The protective role of sulphur dioxide is due to its ability as antioxidant. In the case of the anthocyanins, SO_2 protects the non-coloured anthocyanin in solution until, due to the reversible nature of this reactions, the red anthocyanin chromophore is liberated.

Phenolic acids in grapes and wines include both hydroxycinnamic and hydoxybenzoic acids. Hydroxybenzoic acids, such as gallic acid, show a single intense absorption band at 280 nm, common to all phenolic substances (**Figure 4d**). On the other hand, hydroxycinnamic acids show an absorption band around 320 nm, characteristic of this group of compounds (**Figure 4e** (caftaric acid) and f (coutaric acid)). Finally, the flavonol group show also particular UV-visible absorption features with an additional absorption band around 360 nm (**Figure 4g** (rutin) and h (quercetin)). This absorption band together with the 280 nm absorption features define the UV-visible spectra of the flavonol group.

Grape phenolic compounds are released into the must after the crushing operation. Phenolic compounds are initially located in the solid parts of the berries. Seeds, skins and to a lesser extent, stems, are the main sources of phenolic compounds found in wines. During crushing the juice contained in the berries comes in contact with skins and seeds. Subsequently, during the maceration step this contact will lead to the diffusion of the phenolic substances into the must. While tannins are found in both skins and seed tissues, the anthocyanins are only located in the skins (also found in the flesh of a few tenturier cultivars). Hydroxycinnamic acids are, on the contrary, the only group of phenolic compounds that is found in high levels in the flesh, whereas hydroxybenzoic acids (seeds), stilbenes (skins) and flavonols (skins) are found mainly in the solid parts of the grape berries [4]. The winemaking strategy i.e. presence or absence of skin contact, length and conditions of the skin contact and grape characteristics will define the pool of phenolic compounds that will be present in the wine after the fermentation. Due to this phenolic extraction, important changes in the UV-visible spectral feature take place.

Figure 5 shows the average UV-visible spectral features of 13 different red wines during the first 15 days of the fermentation that included cultivars such as Cabernet Sauvignon, Shiraz, Grenache or Pinotage. Three main absorption bands are observed in the UV-visible spectral features. The first and more prominent band is observed around 280 nm. Following this, broad high intensity absorption properties are also observed around 320 nm. Finally, a third intense absorption band is identified in the visible region around 520 nm. As can be observed in **Figure 5**, right after crushing (Day 0) low absorption intensity bands are observed in the 280 and 320 regions,

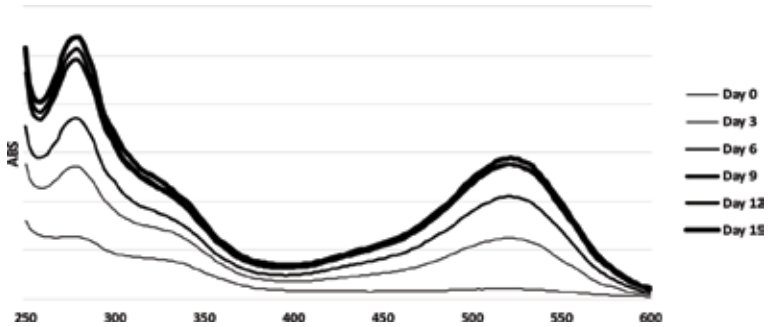

Figure 5.
UV-visible spectral features (250–600 nm) of red wines during fermentation.

whereas no absorption is observed at the visible anthocyanin absorbing 520 nm region. This can be explained by the instant release of some of the phenolic compounds located in the flesh such as the hydroxycinnamic acids. As fermentation progresses a hyperchromic effect is rapidly observed during the first days after crushing. The absorption band around 280 nm rapidly increases until Day 9 of fermentation. From then on, an increase is still identified but to a lesser extent than that initially observed. A different behaviour is observed for the absorption features around 320 and 520 nm. For these two regions, the intense hyperchromism is observed until Day 9 with no subsequent significant increase until the completion of fermentation.

Anthocyanins are water soluble compounds that are extracted during the early stages of fermentation. Alongside with the anthocyanins, the extraction of other skin-localised phenolics, such as flavonols and flavanols or tannins also takes place. However, as alcohol content increases, seed phenolics, mainly flavanols and tannins, are released into the must. The later extraction of seed flavanols and tannins requires the hydrolysis of the lipidic layer around the seed as well as the hydration of the seed tissue itself. Seed tannins have been defined as more astringent and bitter tannins while skin tannins have been described as softer or less reactive towards proteins [10]. The flavanol content in terms of individual composition (procyanidins or prodelphinidins), galloylated subunits and mean degree of polymerisation will provide the intensity and sub qualities of the bitterness and astringency perception of wines [9]. The intense absorption band at 280 nm is due to the extraction of flavonols, hydroxycinnamic acids, flavanols and the UV absorption part of the anthocyanins. The band observed around 320 nm is purely ascribed to the hydroxycinnamic acids. Finally, the band observed at 520 nm is due to the anthocyanin extraction during fermentation. The further increase in the absorption intensity at 280 nm after Day 9 may be due to further extraction of seed tannin content material.

Phenolic compounds are highly reactive and a large number of interactions and reactions can take place during wine ageing and storage. Some of these phenolic reactions benefit from the presence of oxygen during the barrel ageing period. This is the case for some of the direct tannin-anthocyanin complexes as well as the indirectly acetaldehyde mediated tannin-anthocyanin reactions. On the other hand, the absence or shortage of oxygen during the bottle ageing period will stimulate tannin polymerisation reactions and also some direct tannin-anthocyanin combinations. **Figure 6** shows the average UV-visible spectra of a number of commercial red wines after the fermentation process was completed as well as after a year of barrel ageing (12 months after fermentation is completed), followed by a year of bottle ageing (24 months of fermentation completion). In this case an average spectra of a large number of wines including Cabernet Sauvignon, Pinotage, Shiraz, Merlot,

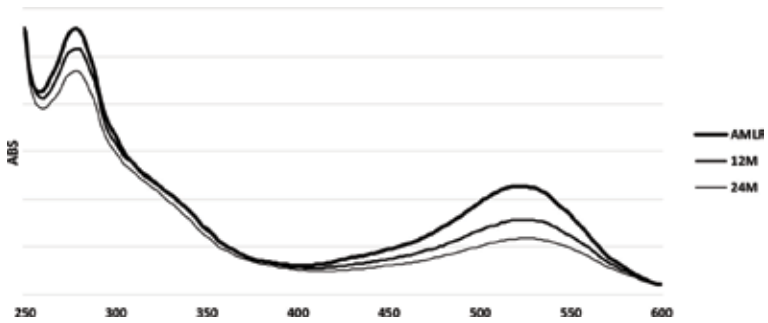

Figure 6.
UV-visible spectral features of wines after malolactic fermentation completion (AMLF) and at 12 months of barrel ageing (12M), followed by 12 months of bottle ageing (24M).

Ruby Cabernet, Petit Verdot, Cinsault, Malbec, Grenache, Pinot Noir and Cabernet Franc was evaluated. The most important features are observed at 280 and 520 nm, whereas the broad band at 320 nm remained constant over the ageing period. It is also important to mention that the bigger decrease in absorption intensity was observed at the 520 nm region which corresponds to the visible absorption part of the anthocyanins.

After reaching maximum levels during the fermentation process, anthocyanin content starts decreasing. Anthocyanins are involved in a large number of phenomena, such as degradation, oxidation, reabsorption into grape and yeast cell walls, precipitation with tartaric salts, interaction with SO_2 or reaction with tannins, among others [7]. Despite this, red wines still maintain an intense colour during ageing which is due to the transformation of anthocyanins into longer term stable polymeric pigments. Anthocyanins give rise to a number of pigments from acylation with diverse grape components, intra and intermolecular copigmentation reactions and interactions, occurring early during the process, to more complex reaction leading to pyranoanthocyanin or tannin-anthocyanin complexes formation [7]. The limited decrease observed around 280 nm is attributed to a larger extent to the decrease of the UV absorption ability of the anthocyanins and to a lesser extent to a decrease of tannin compounds through precipitation. Tannins are also highly reactive substances with high affinity for proteins and polysaccharides, which can lead to tannins precipitation. In addition, the polymerisation ability of these compounds may result in insoluble larger molecules that also precipitate from solution, thus reducing its content in wine. Finally, the absorption band around 320 nm remains stable during ageing, indicating stability of this region absorbing compounds during barrel and bottle ageing.

3. Spectrophotometric methods for phenolic analysis

3.1 Total phenolic content

Total phenolic index (TPI). The measurement of UV-visible absorption light to quantify phenolic compounds was first proposed in the late 1950's. The absorbance at 280 nm was selected as the best indicator of the phenolic content in wine due to the ability of phenolic substances, and more specifically the phenolic ring, to absorb UV light [20]. A simple wine or grape extract dilution is used to quantify the total phenolic content or total phenolic index (TPI). The TPI corresponds to the A280 nm times the dilution factor. The dilution factor might change depending on the sample under evaluation, as well as the path length of the cuvette. Dilution factors of 100

and 50 have been reported for red wines. Depending on the extraction methods dilution factors between 50 and 20 for grape extracts have been proposed. In the case of white and rose wines, with lower phenolic levels, the dilution factor needs to be adjusted. In this case dilution factors from 5 to 20 have been used. The TPI can also be expressed as gallic acid equivalents when used as a standard. This method has been reported to be simple, fast and reliable, although overestimation of the total phenolic content occurs due to the ability of other grape component that also absorb UV-light. A value of 4 units, that can be subtracted from the index, has been proposed to account for the interferences caused by these other UV absorbing material. Additionally, some other phenolic compounds such as cinnamic acids or chalcones do not show absorption features at 280 nm, however due to its low content in wines the expected differences are considered negligible [21].

Folin-Ciocalteu. The Folin-Ciocalteu assay for total phenolic content relies on the ability of the Folin-Ciocalteu reagent to strongly react with phenolic compounds. A mixture of two acids, namely phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMO_{12}O_{40}$) acids, react with mono and dihydroxylated phenolic substances due to their high ability to donate electrons. This reaction creates a blue coloured complex that is quantified at 750 nm [22]. After a simple wine dilution, the Folin-Ciocalteu reagent is added. A 20% $NaCO_3$ solution is then added to the mixture with some additional distilled water. The sample is then incubated for 30 min before absorbance measurement can be performed. Moreover, it is of crucial importance to maintain the order of the additions to ensure that the reaction takes place under alkaline conditions. In order to preserve accuracy, the A750 nm needs to be around 0.3 A.U. If this is not achieved, a different wine dilution needs to be performed. A blank with distilled water, to account for background interferences, is also included [23]. The results are commonly reported as gallic acid equivalents. Although the method is very often used, the ability of some other wine component to also donate electrons leads to potential overestimation of the phenolic content. This compromises the comparison of different samples containing varying phenolic and wine composition. In addition, the comparison of the Folin-Ciocalteu with the TPI is also possible by multiplying the A750 nm times the dilution factor times 20. A strong correlation between the two methods has been reported, thus making the total phenolic content between these two methods comparable [1].

3.2 Total anthocyanin content

Hydrochloric acid method. The estimation of the total concentration of anthocyanins in wine or grape extracts is possible due to the characteristic absorption band of this group of compounds around 520 nm. The coloration of anthocyanins are highly influenced by pH, with lower pH values leading to a higher proportion of anthocyanins in the red flavilium ion form. This property is thus exploited in this method to quantify the total anthocyanin content. Due to its ability to decrease pH, the method makes use of hydrochloric acid (HCl) i.e. the sample is diluted with a 1 M HCl solution. After a waiting period, to allow the free monomeric forms of the anthocyanin to be transformed into their red coloured forms, the A520 nm is measured [24]. The waiting period was initially reported to be longer than 3 hours but shorter than 24 hours, however later research confirmed that a waiting period of 1 hour is sufficient [25]. The values can be reported as A.U. or as malvidin-3-glucoside equivalents by making use of the molar extinction coefficient (commonly used 28,000 L/cm*mol) and the molecular weight (MW = 529 g/mol) of the major anthocyanin found in grapes and wines i.e. malvidin-3-glucoside.

Bisulphite bleaching method. Another property of the anthocyanins is in this case used to quantify this group of compounds. Sulphur dioxide is able to combine with the anthocyanin in the position 4 of the central phenolic ring, giving rise to a non-coloured flavene sulphonate. The decolouration ability of SO_2 is thus used to estimate the total content of free anthocyanins in the wine. The method also makes use of HCl with the aim of transforming the anthocyanins to their red coloured flavilium form. Two test samples are in this case compared. The control sample, with no SO_2 addition is compared against a treatment sample where the anthocyanins have been bleached by the SO_2 addition. After a waiting period, the A520 nm of both samples are compared and the total anthocyanin content calculated [26]. However, the ability of SO_2 to react and bleach some pigmented forms might lead to an overestimation of the total content [27].

pH differential method. Another method that exploits the effect of pH on the anthocyanin coloration was reported by Giusti and Worldstad [17]. This methodology compares a red flavilium form sample at pH 1 against a sample where the anthocyanins are transformed to its non-coloured hemiketal from at pH 4.5. Instead of measuring the anthocyanin content of both pH 1 and pH 4.5 samples at a fixed wavenumber (520 nm), the method measures the A_{max} observed around the 520 nm absorption band, which may not coincide with 520 nm. In addition, the method also includes the measurement of the A700 nm that is subtracted from the A_{max}, with the aim of accounting for possible light scattering caused by other sample components. By doing this the method ensures that the recorded absorption values only correspond to the anthocyanin content in the samples. The results are reported as malvidin-3-glucoside equivalents, by also using the molar extinction coefficient and the molecular weight of this anthocyanin. In addition, the method also allows for the calculation of additional indices by using the ability of sulphur dioxide to combine and bleach anthocyanins. A more complete picture of the anthocyanin content and composition is thus obtained after the inclusion of the pigment degradation, polymeric colour and browning indexes. In this case the method makes use of the absorbance at 420 nm to account for the polymeric anthocyanin material with colour properties closer to this region of the visible spectrum (orange colouration). The polymeric pigment colour is calculated as the proportion between the colour observed in the bleached samples at 420 nm and the A_{max} around 520 nm and that measured at the same wavelengths in the non-bleached samples. In order to ensure accuracy, measurements need to be taken between 15 min and 1 hour in line with what was reported earlier to avoid increased absorption properties at longer times [17].

Modified Somers assay. This methodology is based in the original method reported by Somers and Evans [21]. More recently a modified protocol, adapted to a high throughput format, using a microplate reader spectrophotometer was reported for both grape extract and wine samples [28]. The method presents a number of parameters and provides a broad overview of the status of the anthocyanin's equilibria in the sample. The method relies on the effect of hydrochloric acid, acetaldehyde and sulphur dioxide on the anthocyanins. Sulphur dioxide is added with the aim of calculating the levels of non-blanchable pigments, which includes more stable pigments such as tannin-anthocyanin complexes as well as pyranoanthocyanins. Moreover, acetaldehyde is used to negate the bleaching effect of SO_2 on anthocyanins and thus measure the total content of coloured anthocyanins. Finally, hydrochloric acid is added to account for those free anthocyanins that were not bleached or were derived from copigmentation complexes. The main advantage of the method relies on the fact that the pH adjustment, crucial to accurately estimate the state of the anthocyanins, is done by adjusting the pH of a buffer solution [28]. In the original protocol the pH of the samples was individually adjusted, with a

considerable extension of the time of analysis. This method provides information on the wine "chemical age", which provides an estimation of the extent that the polymeric pigments has displaced the monomeric anthocyanins. Additional parameters report on the percentage of anthocyanins in its flavilium red form (% of ionisation), SO_2 resistant pigments (polymeric pigments), colour intensity, hue as well as total phenolic content.

Copigmentation assay. Anthocyanins interact with other wine components including other phenolic substances to form pigmented molecules through weak hydrophobic forces. The sandwich-like structure is composed of copigment molecules in between the anthocyanins [29]. The newly formed structure places the sugar moieties of the anthocyanin towards the external part of the complex, thus protecting the copigmented pigment from decolouration by water. These interactions account for a large part of the colour of young red wines with its contribution to wine colour decreasing over time, due to the weak nature of the copigmented structure [29]. Two main effects are characteristic of these complexes, which includes an increased absorption intensity in the visible absorption region of the anthocyanins (hyperchromic effect) accompanied by a shift into the absorption maxima towards higher wavelengths (blue colouration) through a bathochromic effect. The copigmentation assay was developed by Boulton and it is the only available method for the quantification of the colour due to copigmentation in red wines. The method relies on the ability of the anthocyanin complexes to avoid decolouration by water at constant pH i.e. measures the decolouration of the anthocyanins due to the dissociation of the copigmented forms [29].

3.3 Colour measurements

Colour density. Coloured anthocyanins and anthocyanin derived pigments are responsible for the colour properties of red wines. During the early stages of winemaking the colour properties of wines are mainly due to less complex monomeric forms of anthocyanins, however as the wine ages and anthocyanins start interacting with other wine components, more stable pigmented polymeric forms are responsible for the colour properties of red wines. The wine colour density was initially measured through the addition of the absorption values at 420 and 520 nm, which corresponds to the yellow and red colorations of wine [30]. Using this information, the hue of a wine samples was defined as the ratio between these two absorption values (A420 nm/A520 nm). More recently the absorption at 620 nm, which accounts for the blue wine colouration, was also added to the colour density parameter [31]. The method relies on a simple measurement (without dilution) and provides an estimation of the colour intensity of the wine. The results are often reported as %yellow, %red and %blue providing thus a more complete interpretation of wine colour properties. On the other hand, the A420 nm or A440 nm are commonly used to measure the colour properties of white wines including the brownish wine colour (browning index) [32].

CIElab colour space. Wine colour can also be measured through the information contained in the visible spectra of wines. Three colour components result from the integration of the visible absorption features. The Commission International de l'eclairage [33] proposed a method that uses three chromatic coordinates X, Y and Z to determine the chromatic characteristics of wines (also applicable to other beverages). The method aims to simulate the perception that real observers have for the colour properties of a sample. The calculation of the CIElab coordinates is based on measurement conditions given by a spectrophotometer with illuminant D65 and observed placed at 10°. The colour of a wine is thus described by the intensity of the wine colour (chromaticism), the luminosity of the wine and the colour itself based on the

red, yellow, green and blue components (tonality). The colorimetric measurements are defined by the chromatic coordinates red/green component (a^*) ($a^* > 0$ red, $a^* < 0$ green), blue/yellow component (b^*) ($b^* > 0$ yellow, $b^* < 0$ blue), clarity (L^*) ($L^* = 0$ black and $L^* = 100$ colourless) and its complementary magnitudes tone (H^*) and chroma (C^*). The ability to compare the colorimetric differences between two colours (ΔE^*) makes it possible to directly compare the colour properties of wines. Moreover, it has been established that a colour difference higher than 2.7 indicates that the colour of two samples can be perceived different by the human eye [34].

3.4 Total tannin content

Acid hydrolysis. Due to the complex nature of proanthocyanidins or tannins the determination of these compounds is a difficult undertaking and has been challenging researchers for a long time. However, a number of methods, albeit with certain limitations, have been reported and will be discussed. The acid hydrolysis method is based on the transformation of proanthocyanidins in carbocations that are partially converted into anthocyanidins when exposed to heating under acidic conditions (Bate-Smith reaction). The total tannin content is thus estimated by using the red coloration of the resulting anthocyanin compounds at 550 nm and expressing it in cyaniding-3-glucoside equivalents. Although the method is widely used, a number of limitations have also been reported. First of all, the tannin concentration seems to be overestimated with higher values for tannins reported than those for total phenolic content. Moreover, it is also common to observe an increase in the total tannin content of wine during ageing and finally the method does not provide any information on the structure of the tannins [35].

Methylcellulose precipitable (MCP) tannins assay. This method falls under the precipitation based methods category as it uses the tannin precipitation ability of a methylcellulose polymer to estimate the total tannin content of grape extracts and wines. As mentioned the method relies on tannin-MCP interactions in the presence of ammonium sulphate, giving rise to an insoluble polymer-tannin complex that precipitates and is further separated by centrifugation [36]. This method has also been lately adapted and validated into a high throughput format leading to a considerable reduction of the analytical time [28]. A control sample without MCP addition (absence of tannin precipitation) is compared against a treated sample where the tannins have been removed after precipitation with MCP. The absorption difference measured at 280 nm is then used to quantify the total tannin content of a sample. The total tannin content is in this case estimated as epicatechin equivalents. In addition, one of the main benefits of precipitation based methods is that a theoretic positive correlation with astringency intensity is foreseen [37–39]. The hypothesis is based on the assumption that the method simulates the phenomena that naturally occurs in wine when it becomes in contact with the salivary proteins. An insoluble macromolecular complex is then formed that precipitates from solution causing the drying and puckering sensation known as astringency.

Bovine serum albumin (BSA) tannin assay. This precipitation based method exploits the ability of proteins to combine and precipitate tannins. The precipitation is achieved through the incorporation of bovine serum albumin protein. The precipitated protein-tannin complexes are then redissolved and quantified at 510 nm after the addition of ferric chloride [40, 41]. The accuracy of the method is based on obtaining the appropriate wine dilution as concentrated or very diluted samples tend to underestimate the tannin content. The BSA tannin assay, as part of the precipitation based methods for tannin analysis, has also been found to positively correlate with astringency intensities given by sensorial evaluation [37–39]. The total tannin content is in this assay calculated as catechin equivalents. In addition,

the method also allows for the determination of additional parameters related to the anthocyanin and polymeric pigment fraction. Specifically, the method makes use of SO_2 to obtain information on the nature of the polymeric pigments by dividing them into small (SPP) (pigments that do not precipitate with BSA) and large polymeric pigments (LPP) that do precipitate with the protein. On the other hand, the comparison of both precipitations based methods has shown that MCP tannin values are on average three time higher than those found for BSA. However, a strong correlation (0.8) between the values obtained with the two methods has also been reported [42], whereas no correlation was observed between these two methods and the tannin content obtained with the acid hydrolysis method [37]. Finally, despite the differences in absolute values, attributable to the differences in both procedures, it has also been stated that both precipitants (BSA and MCP) precipitate the same amount of tannins when tested under the same conditions [39, 43].

4. UV-visible role in liquid chromatography

High liquid pressure chromatography (HPLC) is a suitable method to quantify individual phenolic compounds in grape extracts and wines. HPLC instruments make use of a diode array detector that allows for the quantification of phenolic substances at different wavelength within the UV-visible regions. The benefit of using diode array detectors in liquid chromatography is beyond using retention times for peak identification as it adds qualitative information by the incorporation of the UV-visible spectral features of a specific peak or compound [44]. It is thus nowadays possible to obtain a number of individual phenolic compounds by direct injection of wine samples without any sample pre-treatment. Based on its spectral features, phenolic compounds will be quantified at their absorption maxima, i.e. sub-families of phenolics are quantified at 280 nm for flavanol monomers and polymers and some phenolic acids, 320 nm for hydroxicinnamic acids, 360 nm for flavonols and finally 520 nm for anthocyanins. Although a considerable number of individual phenolics can be quantified using HPLC, the majority of the methods are not able to separate larger molecular structures such as polymeric phenols and pigments [13]. These two groups of compounds are commonly identified as broad absorption bands at later elution times at 280 nm for the polymeric phenols and at 520 nm for the polymeric pigments. Furthermore, in a previous study, the composition of the broad absorption band observed at 520 nm theoretically attributed to polymeric pigment material was investigated and confirmed [13]. Additionally, the polymeric pigments peak was also found to correlate with the spectrophotometric measurements of phenolic compounds and with wine age. In terms of polymeric phenols, it is believed that the phenolic compounds forming part of this broad absorption band correspond to a large extent to proanthocyanidins or tannins of high degree of polymerisation. The strong correlation (0.83) observed for a significant number of wines between the polymeric phenol peak area and the total tannin content, obtained with the MCP tannins assay, confirmed this [16]. HPLC methods for quantification of phenolic substances can also incorporate mass spectrometers. Mass spectrometry provides information about the molecular weight of the compounds and it is used to discern the identity of unknown compounds. The identification of phenolic compounds in chromatographic techniques using DAD is limited by co-elution (impure UV-visible spectra) or by similarities in the UV-visible properties of phenolic compounds belonging to the same phenolic family. These factors combined with similar elution times of some of the phenolic substances complicates the accurate quantification of chromatographic peaks. The use of mass-spectrometry provides thus a valid tool to confirm the identity of phenolic substances as well as the identification of novel compounds.

5. Fluorescence spectroscopy

An interesting and more recent technique to quantify phenolic compounds makes use of the ability of this group of substances to emit fluorescence light after the excitation/emission process. Fluorescence spectroscopy is able to measure the analyte concentration through its fluorescence properties, being thus suitable to measure compounds in solution, such as phenolics found in grape extracts or wines [14]. If phenolic compounds are excited at the appropriate light intensity and wavelength, generally through UV light exposure, the energy change occurring at electronic level will cause a light emission in the visible region of the electromagnetic spectrum [45]. Phenolic molecules are initially at ground levels at low energy state until light exposure elevate the vibrational levels to an elevated high energy state. After a period of time (in the order of milliseconds) the excited molecule while returning to its non-excited electronic state emits light (so-called fluorescence) at higher wavelengths than those absorbed during the excitation process. During the excitation/emission sequence both the absorbed and emitted light can be measured, with higher emission intensity corresponding to higher concentration of the analyte. Fluorescence spectroscopy has been commonly applied to the quantification of phenolic compounds in combination with liquid chromatography techniques. The main benefit of these applications rely on the increased sensitivity and selectivity of the method [45]. Additionally, fluorescence spectroscopy has been defined as a fast, non-destructive, easy to perform technique that can also be used for process monitoring purposes due to the versatility of the fluorescence spectrometers. Excitation emission spectral (EEM) properties might potentially be correlated with reference analytical data to establish regression calibrations for the quantification of phenolic compounds in a similar manner than what is reported for UV-visible or infrared spectroscopy calibrations.

6. UV-visible spectroscopy with chemometrics

The UV-visible spectra can alternatively be used in combination with powerful chemometric analysis to obtain spectroscopic calibrations for the prediction of phenolic content in grapes as well as in wines during the winemaking process [15]. In this case the totality or parts of the UV-visible spectra are correlated through multivariate regression approaches with reference phenolic data. After the spectral and phenolic content acquisition of a significant number of samples and in the case that strong correlations are found between the spectral data and the phenolic levels, a reliable prediction calibration can be obtained after the corresponding calibration and validation procedure. The advantage of these spectroscopy calibrations relies on the possibility of estimating the total content of phenolic substances through a simple spectral measurement, therefore avoiding the tedious reference method procedures. The main advantage of the spectroscopy calibrations is due to the rapidness, simplicity, reliability and cost-effectiveness ascribed to these techniques. Moreover, due to the multi-parametric nature of this approach a single spectral measurement is able to provide the levels of a number of phenolic compounds. Spectroscopic applications are also highly suitable to perform online measurement during the process of winemaking, allowing for improved process control strategies, through process monitoring, in line with a process analytical technologies (PAT) approach [46].

The first indication of the use of UV-visible spectroscopy calibrations to quantify some of the most important phenolic parameters was reported in 1995 [47]. In this first approach the total tannin and anthocyanin content was predicted using

a limited number of samples. The UV-visible spectra was collected from 200 to 650 nm at 6 nm intervals. Errors in prediction (root mean standard error of prediction (RMSEP)) of 0.35 g/L (14% RMSEP%) and 29 mg/L (8%) where reported for tannins and anthocyanins respectively. Despite the relative small sample set and the limitations of the analytical reference method investigated (mainly due to non-specificity for phenolic compounds of the employed procedures) this publication reported for the first time the suitability of UV-visible spectroscopy to quantify phenolic content in wines through partial least squares (PLS) regression analysis.

In a further study the UV-visible spectral properties of a large dataset (400) were used to quantify phenolic content of samples collected at different stages of the winemaking process. The sample set included samples from a variety of different regions and cultivars. Spectral data was collected over the 230–900 nm at 0.17 nm intervals. The parameters derived from the BSA tannin assay including the anthocyanin and total phenolic related parameters were in this case evaluated. RMSEP of 87 mg/L (20% RMSEP%) for total anthocyanin content; 0.37 (26.4%), 0.46 (76.7%) and 0.48 (24%) A.U. for small, large and total polymeric pigments, respectively; 66 mg/L (30.1%) for tannin content; 99 (17.2%) mg/L for non-tannin phenols and 130 mg/L (16.4%) for total phenols were reported [48]. Later on, the same phenolic parameters were again investigated. A 100 samples of Cabernet Sauvignon and 100 samples of Shiraz were collected during the fermentation process over a single vintage to provide calibration that can be used for the prediction of phenolic content in must. In this case an adaptation of the BSA tannin assay was used for phenolic analysis. UV-visible spectral properties were collected in the 200–900 nm range. The results showed calibrations able to predict the phenolic content of Cabernet Sauvignon samples, but not for Shiraz, suggesting cultivar specificity of the predicted calibrations. Standard errors in cross validation (RMSECV) of 102.22 mg/L (23.8% RMSECV%) and 211.38 mg/L (25.6%) were reported for total tannin and iron reactive phenolic content, respectively for Cabernet Sauvignon samples. In terms of anthocyanin measurements, error in cross validation of 101 mg/L (43.3% RMSECV%), 0.46 A.U. (26.1%) and 0.48 A.U. (41.4%) for total anthocyanins, small and large polymeric pigments were observed [49].

Due to its characteristic absorption band at 280 nm the UV spectral properties of wines have also been used for the determination of phenolic content and more specifically for total tannin content. The MCP tannin levels of a significant number of samples from a variety of different locations, cultivars and during different steps of the winemaking process were successfully predicted with the use of multiple linear regression (MLR) and partial least square regression (PLS). In this case spectral properties were collected between the 230 and 350 nm range of the UV part of the electromagnetic spectrum. Errors in cross validation (RMSECV) of 0.2 g/L (9.3% RMSECV%) were reported for MLR models using the above-mentioned UV region. Moreover, the authors also reported calibrations but in this case using only a limited number of key wavelengths. Further calibrations were investigated using the UV absorption values at 250, 270, 280, 290 and 315 nm. The external validation calibrations showed errors in prediction (RMSEP) of 0.18 g/L (9.2%) which confirmed the suitability of the UV region spectral properties to quantify tannin content in wine samples [50].

In a more recent study the ability of UV-visible spectroscopy to predict tannin content in finished wines was reported. In this case two precipitation based methods, namely MCP and BSA tannin assays, were used to generate the spectral data. A large number of samples containing a varying number of cultivars from different regions as well as vintages were included in the model optimization procedure. UV-vis spectra was measured in the 260–610 nm region at 2 nm intervals. The best

calibrations were found for both reference methods where the spectral properties in the UV region were used as spectral data (260–310 nm). A RMSEP of 0.16 g/L (9.9% RMSEP%) and 0.08 g/L (13.3%) were reported for MCP and BSA tannin content, respectively. In agreement with previous studies, accurate calibrations were also observed when a reduced number of wavelength were used as spectral data. Models optimised using the absorption values at 270, 280, 290, 300 and 314 nm lead to errors in prediction of 0.18 g/L (11.2% RMSEP%) and 0.11 g/L (18.3%) for MCP and BSA tannin content, respectively. Also in agreement with previous findings, cultivar and vintage specificity issues influenced to a certain extent the accuracy of the calibrations [42].

In a more recent study PLS calibrations based on UV-visible spectral data for the quantification of phenolic content in grapes, fermenting samples and wines have been reported. A large number of fermenting samples from 13 different vinifications over two consecutive vintages were included into the calibrations. Moreover, a number of finished wines from varying vintages and from a number of cultivars were also included. PLS validation calibrations showed prediction errors of 209 mg/L (14.3% RMSEP%), 14 mg/L (3.2%), 1.6 (3.2%) and 2.6 (14.7%) for total tannin content (MCP tannin assay), total anthocyanins, total phenolic content and colour density, respectively. In addition, individual phenolic compounds quantified using a HPLC method to generate the reference data were also reported, including flavanol monomers and the dimer B1, phenolic acids, flavonols as well as monomeric and acylated anthocyanins. Calibration for the estimation of polymeric phenol and pigment content were also reported. On the other hand, the same study reported PLS calibrations for determination of phenolic content in grapes extracts obtained through two extraction protocols. A phenolic extraction in high solvent content and after the entire berries being finely blended lead to successful calibrations for total tannin content, anthocyanins levels, total phenol index and colour density. The RMSEP reported was 0.22 mg/g (7% RMSEP%), 0.034 mg/g (3.1%), 0.17 (1.32%) and 0.72 (6.61%), for the above mentioned parameters, respectively. In addition, an alternative method with phenolic extraction performed under wine-like ethanol levels from hand crushed grapes was also reported. Validation errors of 0.12 mg/g (10.7% RMSEP%), 0.03 mg/g (8.33%), 0.42 (1%) and 6.2 (20%) for total tannins, total anthocyanins, total phenolic index and colour density, respectively were reported [16].

7. Conclusions

The role of UV-visible spectroscopy in wine science appears to be of high importance. A number of applications can be used to quantify the levels of phenolic compounds in grape extracts and wines. Apart from the conventional routine spectrophotometric methods for phenolic analysis, more advanced analytical techniques such as liquid chromatography can be also used to quantify individual phenolic substances using UV-visible spectroscopy. Moreover, fluorescence spectroscopy, making use of the ability of phenolic molecules to emit fluorescence light, appears to be a promising technique that can also be used to quantify phenolic content at different stages of the winemaking process and under different conditions. Finally, UV-visible spectroscopy calibrations are also a valid alternative as they allow for the efficient measurement of phenolics in grape extracts as well as wines during fermentation and ageing. These new developments in phenolic monitoring during the winemaking process opens exciting new possibilities for wine producers in their bid to obtain wines of a certain composition and style in a more controlled manner.

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Chapter 4

Probiotic, Prebiotic and Synbiotic Products in Human Health

Nicoleta-Maricica Maftei

Abstract

The health benefits imparted by probiotics and prebiotics as well as synbiotics have been the subject of extensive research in the past few decades. What is the real role of probiotics strains, prebiotics and synbiotics in influencing a health? To battle the increase in health care costs, in recent years has been developed a preventive approach to medicine with the development of new probiotics and prebiotics or symbiotic products. Many studies suggest that probiotics, prebiotics and synbiotics supplementation may be beneficial in prevention and management of nutritional and health. While these studies show promising beneficial effects, the long-term risks or health benefits of prebiotics, probiotics and synbiotics supplementation are not clear. In this chapter review the literature regarding available information and summarises the current knowledge on the effects of probiotics, prebiotics, and synbiotics on human health and explore recent trends and developments in this field.

Keywords: probiotics, prebiotics, synbiotics, health, functional food

1. Introduction

Probiotic, prebiotic and synbiotic are words of the modern era, bookmark "for life" and is in use to define bacterial association with beneficial effects on human health. In the world of highly processed food, both at the industrial and nutritional level clear consideration are paid to the composition and safety of the intake products. The nutrition quality is essential for human health because of the food poisoning, obesity, allergy, cardiovascular diseases, and cancer, that is consider the plague of the twenty-first century. Worldwide, many research reports underline the health advantages of using probiotics, prebiotics and also, synbiotics in human consumption [1]. In early 1990s, Metchnikoff [2] defined probiotics in a scientific context as the microorganisms that alter of floral/microbial diversity in human bodies and replaces the harmful microbes with useful ones. However, Tissier detected that the microbial population of a particular type of bacteria in stool samples of infected diarrheic children was significantly lower comparing to healthy children [3]. He suggested that patients with diarrhoea (infantile diarrhoea) should oral administration of live organisms (bifidobacteria) and in this way a healthy gut flora was restored. Havenaar and Huis in't Veld [4] have given the modern definition of probiotic: as a viable mono or mixed culture of bacteria which, when applied to animal or man, affects the host beneficially by improving the properties of the indigenous flora. In 2002, Food and Agriculture Organisation of the United Nations (FAO) and World Health Organisation (WHO) defined probiotics as being "live strains of strictly selected microorganisms which, when administered in adequate

amounts, confer a health benefit on the host" [5]. The definition was preserved also, by the International Scientific Association for Probiotics and Prebiotics (ISAPP) in 2013 [6]. The vast majority of results of the clinical research underline the positive effect of the probiotics on the gastrointestinal diseases, such as: irritable bowel syndrome, gastrointestinal disorders, elimination of *Helicobacter*, inflammatory bowel disease, diarrhoeas, and allergic diseases, like as atopic dermatitis. Also, numerous clinical reports have demonstrated the efficiency of the probiotics for the treatment of diseases such as obesity, insulin resistance syndrome, type 2 diabetes, and non-alcoholic fatty liver disease. Increasing the body's immunity (immunomodulation) was the positive effect of probiotics on human health. Majority of scientific reports also show the benefits of the prophylactic use of probiotics in different types of cancer and side effects associated with cancer [1].

In 1995, Gibson and Roberfroid defined prebiotics were by as non-digested food components that, through the stimulation of growth and/or activity of a single type or a limited amount of microorganisms which residing in the gastrointestinal tract, improve the health condition of a host [7]. Instead, in 2004, prebiotics were described as selectively fermented compounds permitting precise changes in the composition and/or activity of the gastrointestinal tract microorganisms, these changes being useful for the host's health and wellbeing [8]. Recently, in 2007, FAO/WHO experts, designated prebiotics as a nonviable food constituent that confers a health advantage on the host linked to the microbiota modulation [9]. However, in the literature it is specified that prebiotics can be used as a probiotics substitute or as a supplementary support for them. Instead, numerous prebiotics can improve the growth of indigenous gut bacteria and have tremendous potential for changing the gut microbiota, but these variations occur just at the level of individual strains. Worldwide, numerous scientific studies underline the positively effects of the prebiotics for human health.

For the simultaneous use of probiotics and prebiotics high potential is attributed. In 1995, Gibson and Roberfroid introduced the term "synbiotic" to describe union between probiotics and prebiotics synergistically acting of health [7]. Synbiotic is a designated compound that introduced in the gastrointestinal tract can careful stimulates the growth and/or activates the metabolism of physiological intestinal microbiota, thus conferring beneficial result to the host's health [10]. As the word "synbiotic" is a synergy, the term can be attributed only to the products where a prebiotic compound selectively improves a probiotic microorganism [11]. The main aim of this type of combination is the improvement of probiotic microorganism's survival in the gastrointestinal tract. Therefore, synbiotic have both probiotic and prebiotic assets and were designed in order to solve the probiotics survival in the gastrointestinal tract [12]. An adequate combination of both components (prebiotic and probiotic) in a single product should guarantee a superior effect, compared to the action of the probiotic or prebiotic alone [13, 14].

Besides basic role of the nutrition consisting in the supply of necessary nutrients for growth and development of the organism, some additional aspects are becoming increasingly important, including the maintenance of health and counteracting diseases. The introduction of probiotics, prebiotics, or synbiotics into human diet is favourable for the intestinal microbiota and the human health. They may be consumed in the form of dairy products, raw vegetables and fruit or fermented pickles. Another source of probiotics, prebiotics, or synbiotics may be pharmaceutical formulas and functional food. Although probiotics, prebiotics and synbiotics have considerable potential in nutritional and clinical applications, considerable researches are required for the implementation of probiotics into human health, nutrition and regulation of different abnormalities. The screening of probiotics, prebiotics and synbiotics and their amounts is essential in gaining a therapeutic effect in health. However, further research focused on discovering new probiotic strains,

the assortment of probiotics and prebiotics for synbiotics, dose setting, safety of use, and clinical trials is necessary. Also, the health benefits should be established in properly scheduled clinical trials conducted by independent research centres.

This chapter is an attempt to emphasise the possible benefaction of probiotics, prebiotics and synbiotics for improving human health and regulation of common metabolic disorders or abnormalities.

2. Probiotics

Gut bacterial colonisation starts since at birth when new-borns are exposed to a nonsterile climate. Henceforth, it changes and transforms over a lifetime, depends on a complex and dynamic interaction between the diet, genome, and lifestyle of the host, as well as antibiotic consumption. Remarkable bacterial colonisation of age-specific changes described in gut microbiota configuration include a decrease in the Bacteroidetes/Firmicutes ratio and a reduction in bifidobacteria in people aged over 60 years, when the immune system starts to decline [15]. Normally, the composition of the intestinal microflora is considered to be constantly throughout adulthood period.

Since the beginning of the twentieth century the interest in lactic acid fermentation was expressed by the Russian scientist and immunologist, Ilia Miecznikow, that worked at Pasteur Institute, Paris. In the book "Studies on Optimism" he affirmed that "with various foods undergoing lactic acid fermentation and consumed raw (sour milk, kefir, sauerkraut, pickles) humans introduced huge amounts of proliferating lactic acid bacteria to their alimentary tracts" [16].

2.1 Probiotic strains

The microorganisms that are used as probiotics can belong to different types, such as bacteria, yeast and mould. Selected probiotic bacteria strains can be as following:

a. *Lactobacillus: acidophilus*, *sporogenes, plantarum, rhamnosus, delbrueckii, reuteri, fermentum, brevis, casei, farciminis, paracasei, gasseri, crispatus*;

b. *Bifidobacterium*: *bifidum, infantis, adolescentis, longum, thermophilum, breve, lactis;*

c. *Streptococcus*: *lactis, cremoris, thermophilis, diacetylactis*;

d. *Leuconostoc mesenteroides*;

e. *Pediococcus* spp.;

f. *Propionibacterium* spp.;

g. *Enterococcus—Enterococcus faecium*;

The literature mentions as probiotics the following yeast and mould strains:

a. Yeast: *Saccharomyces cerevisiae, Saccharomyces bourlardii*, *Candida pintolopesii, and Sacaromyces boulardii*

b. Moulds: *Aspergillus niger, A. oryzae* [17].

The type of the microbes used as probiotics increased due to the increase in the research concerning the health but as well as by the increase of the newly discovered and identified microbes, which could be used as probiotics in different food and beverages with huge impact on human body.

With the development of better culturing methodologies, more affordable genome and metagenome sequencing, the probiotic research is in a fulminant era, one which permits designing adapted probiotics that address specific consumer needs and issues. Also, the data of the conformation and role of the human gut microbiome accelerated by massively parallel sequencing, has extended the range of microorganisms with possible human benefits, although many of these are still at the very early stage of research.

These organisms are sometimes referred to as next-generation probiotics (NGPs), but may also be termed live biotherapeutic products (LBPs). NPGs obviously follow to the standard classification of a probiotic, but mainly referring to those microorganisms that have not been used as agents to promote health till now, and which are more likely to be delivered under a drug regulatory framework. Next-generation probiotics fit well within the US Food and Drug Administration (FDA) definition of a live biotherapeutic products: "a biological product" that: comprises live microoorganisms, such as bacteria; it is not a vaccine; is applicable to the prevention, treatment, or cure of a disease or condition of human beings [18].

Examples of current NGP: *Faecalibacterium* spp., *Akkermansia* spp., *Bacteroides fragilis* strain ZY-312, *Bacteroides xylanisolvens* DSM 23964, *Clostridium butyricum* MIYAIRI 588, *Faecalibacterium prausnitzii* and other.

Probiotics are subject to regulations in the general food law worldwide, conforming to they should be safe for human and animal health. In the Unite State of America, microorganisms that are used for human consumption should have the Generally Regarded As Safe (GRAS) status, regulated by the Food and Drug Administration (FDA). Rather, in Europe, European Food Safety Authority (EFSA) introduced the term of Qualified Presumption of Safety (QPS). The term of QPS it is a concept which involves some additional criteria of the safety assessment of bacterial supplements, including the history of safe usage and absence of the risk of acquired resistance to antibiotics [19, 20]. Until this moment mechanism of action of probiotics has not been clearly understood, but research results are those obtained from animal models and in vitro experiments. From a medical point of view it is considered that action mode of probiotics may improve the barrier functions of the gut mucosa because several strains of *Lactobacillus* spp. and *Bifidobacterium* spp. as well as structural compounds, and microbial-produced metabolites are able to stimulate epithelial cell signalling pathways. Thomas and Versalovic [21] reported that the Nuclear FactorKappa-Light-Chain-Enhancer of activated B cells (NF-kB) pathway is controlled by probiotics at many different levels with effects seen on I Kappa B protein (IKB) degradation and ubiquitination, proteosome function [22] and nuclear-cytoplasmic movement of RelA through a PPAR-gamma dependent pathway. Also, it is known that probiotics can modulate the immune system functions for instance, *L. acidophilus* has been found to modulate toll-like receptors and the proteoglycan recognition proteins of enterocytes. This thing leads to activation of dendric cells and lymphocytes T-helper 1 responds. After stimulation of lymphocytes T-helper 1 cytokines can suppress lymphocyte T-helper 2 responses which provoke the atopic issues [23]. Another possible mechanism of action of probiotics may be their ability to suppress the growth of pathogenic bacteria by producing broad-spectrum bacteriocins [24]. After the latest research on probiotics we can conclude that molecular and genetic research allowed the determination of the beneficial effect of probiotics, involving four mechanisms:

1. Antagonism through the production of antimicrobial compounds [25];

2. Pathogens competition for adhesion to the epithelium and for nutrients [26];

3. Immunomodulation of the host [27];

4. Inhibition of bacterial toxin production [28].

The first two mechanisms are directly related with their effect on other microorganisms. Nevertheless, all four mechanisms, from medical point of view, play an important role in the infections prophylaxis and treatment and also, for maintenance a balanced host's intestinal microbiota [1]. The capability of probiotic strains to co-aggregate, as one of their mechanisms of action, can contribute to the development of a protective barrier preventing pathogenic bacteria from the colonisation of the gut epithelium [29]. Probiotics bacteria are able to adhere to epithelial cells, inhibiting the pathogens. This mechanism plays an important effect on the host's health condition. Also, the adhesion of probiotic microorganisms to epithelial cells can start a signalling cascade, leading to immunological modulation. Otherwise, the discharge of some soluble compounds may cause a direct or indirect (through epithelial cells) activation of immunological cells [30].

Probiotics may have an significant role in: chronic inflammation of the alimentary tract or of a part thereof, the prevention and treatment of contagious diseases, lactose intolerance and lactose digestion, cholesterol reduction, cardiovascular health, urogenital disease, allergic disease, oral health, gastrointestinal disease, obesity but and an possible role in the elimination of cancer cells.

2.2 Probiotics in human health

2.2.1 Probiotics in prevention and treatment of acute diarrhoea and diarrhoea associated with antibiotics

Diarrhoea induced by antibiotics is a very common complication in the hospital setting, representing a percentage by 13–60% and disease caused by Clostridium difficile is also a significant cause of nosocomial diarrhoea and colitis that prolongs the hospital stay by 3–7 days and increases the risk of new nosocomial infections with 20–65%, costs, and mortality (2- or 3-fold depending on reports) [31]. The roles of the probiotics used to treat these patients are:

1. restoration intestinal microflora;

2. increase immune response;

3. compete with pathogenic bacteria;

4. remove their toxins.

Saccharomyces boulardii, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus rhamnosus, Lactobacillus paracasei, Bifidobacterium longum, Bifidobacterium breve there were some of the most widely studied probiotics for treatment of acute diarrhoea. In a recent meta-analysis of 21 studies (4780 patients), the administration of *S. boulardii* decreased the risk of antibiotic-induced diarrhoea in both children and adults from 19 to 8.5%, with a relative risk of 0.47. In another meta-analysis of 82 randomised clinical trials using diverse species (usually

Lactobacillus spp., alone or combined with bifidobacteria, enterococci, or *S. boulardii*), a reduced risk of antibiotic-induced diarrhoea was also established, with a relative risk of 0.58 [31].

Floch et al. [32] reported that for the primary prevention of disease caused by *C. difficile* in patients treated with antibiotics, probiotics also decrease the incidence of such disease, especially when strains of *S. boulardii*, and possibly other *Lactobacillus*, such as GG, are administered. Instead, a recent meta-analysis settled that only four probiotic strains (not including *Lactobacillus* GG) have been shown to significantly decrease the incidence of diarrhoea induced by *C. difficile*, such as follows: *S. boulardii* (2×10^{10} CFU/day), *L. casei* DN114001 (probiotic drink twice daily), a mixture of *L. acidophilus* and *Bifidobacterium bifidum* (2×10^{10} CFU/day), and a mixture of *L. acidophilus*, *L. casei*, and *L. rhamnosus*) [33].

For patients that intake antibiotics to eradicate *Helicobacter pylori*, studies have been conducted based on probiotics adding in order to increase eradication rates and also to prevent side effects such as antibiotic-induced diarrhoea. Numerous meta-analyses showed that the addition of probiotics may increase the efficacy of eradication with an odds ratio (OR) ranging from 1.2 to 2 times compare to the control group. Although additional studies are needed, it appears that the most effective strains are *L. acidophilus* (1.25×10^9 CFU) (OR: 1.24), milk fermented with *L. casei* DN-114001 (2 packs daily) (OR: 1.47), yogurt with *Lactobacillus gasseri* (OR: 1.19) (2 packs daily), and *Bifidobacterium infantis* (2×10^9 CFU) (OR: 1.21) [31]. Also, in supplementary clinical researches where antibiotics are used, probiotics appear to decrease the incidence of diarrhoea (with an OR ranging from 0.16 to 0.47) [34].

Also, the efficiency of probiotic strains in the next therapy's: nosocomial, non-nosocomial, and viral diarrheas have been studied. The conclusion was as follows: it turns out that probiotics may increase the amount of IgA antibodies, which leads to the decrease number of a viral infection [35].

2.2.2 Probiotics in in diseases of the gastrointestinal apparatus

Inflammatory bowel disease (IBD) is a recurrent chronic condition in which an abnormal interaction exists between intestinal flora and the host. Patients with IBD have an increased risk of colorectal cancer [31]. Due to the growing area of disease spreading and ageing societies, the use of probiotic bacteria for human health is becoming increasingly important. The consumption of pre-processed food (fast food), often containing excessive amounts of fat and insufficient amounts of raw fruits and vegetables, is another factor of harmful modification of human intestinal microbiota. It seems that the system of intestinal microorganisms and its desirable modification with probiotic formulas and products may protect people against enteral problems, and improve health [1]. *L. plantarum* is a probiotic that has been used with good results in the management of some symptoms in patients with IBD. It has been reported that the DSM 9843 strain significantly reduced flatulence, and the LPO 1 and 299 V strains significantly reduced abdominal pain [36]. In many studies it has been reported that probiotics may be helpful in the treatment of inflammatory enteral conditions, Crohn's disease, ulcerative colitis, and non-specific ileitis. The aetiology of those diseases is not completely understood, but it is evident that they are associated with recurrent infections or chronic inflammations of the intestine. Using a complex probiotic, such as: VSL#3, which contains 4 lactobacilli strains—*L. acidophilus*, *L. casei*, *Lactobacillus delbrueckii* sp. *bulgaricus*, and *Lactobacillus plantarum;* 3 bifido bacterial strains: *B. longum*, *B. infantis*, and *Bifidobacterium breve;* and *Streptococcus salivarius* sp. *thermophilus*, revealed to decrease activity of pouchitis (a non-specific inflammation of the ileal pouch) in ulcerative colitis (UC) and after ileal anastomosis [37]. The frequent

doses recommended in pouchitis are 2–4 sachets daily (each sachet contains 450,000 million live bacteria 4.5×10^{11} CFU; but there are also capsules containing 112,000 millions of bacteria) [31]. However, in additional research was described lower improvements in the reduction of disease in association with conventional treatment in patients with UC, and minor to moderate contribution, with the use of probiotic VSL#3, *Escherichia coli* Nissle, *Lactobacillus* GG, or milk fermented with bifidobacteria and/or lactobacilli (whether or not compared to placebo or other treatments, such as mesalazine) [38]. In trials with probiotics on remission induction or maintenance in Crohn's disease (using several strains such as *Lactobacillus* GG, VSL3, *L. johnsonii* LA1, *Escherichia coli* Nissle 1917, *S. boulardii*) have been reported less satisfactory results than in UC [32].

In a 2007 [39] demonstrated that administering probiotics may improve the rate of eradication and reduce the incidence of adverse events in case of infection with *Helicobacter pylori*. Zhang et al. [40] informed that the using the probiotics for standard eradication therapy in patients infected with *H. pylori* may increase the rate of eradication of the microorganism by approximately 13% and decrease the overall rate of adverse effects by approximately 41%, based on the patient's age, gender or probiotics dose. The probiotic used to improve the results of eradication therapies was *Lactobacillus reuteri*. In these therapies which demonstrated an ability to inhibit the colonisation of the human gastric mucosa with *H. pylori*, in addition to an ability to produce reuterin, a broad-spectrum antibiotic active against *H. pylori*, DSM 17648 strain of *L. reuteri* seemed especially effective for eradication therapies [36]. Also, *S. boulardii* seemed to significantly increase the rate of eradication, although under the desired success level (80% versus 71% in the control group) [36].

Few studies are, so far available, and, consequently much clinical evaluation is needed in the future of the most effective strains and of how host factors (such as the genetic characteristics of patients) influence therapeutic response.

2.2.3 Probiotics in liver disease

The researchers reported that probiotics can be useful in treating hepatic diseases due to their potential ability to modulate alterations in the gut microbiota, intestinal permeability, and immune and inflammatory responses. More studies based on murine and *in vitro* models show the role of probiotics in several liver diseases [41]. From medical point of view the pathogenic mechanism involved in liver damage secondary to alcohol abuse is endotoxemia. Researchers reported that through using *L. plantarum* encapsulated alginate beads induce a dose-dependent reduction of endotoxin level in rats exposed to alcohol [41].

Domingo [36] suggests that non-alcoholic fatty liver disease (NAFLD) comprises a varied range of pathological circumstances, from simple steatosis to cirrhosis, through steatohepatitis and fibrosis. It is known that probiotics (VSL#3) can modulate the intestinal flora, influencing the bowel-liver axis and improving NAFLD. Xu et al. [42] reported in a study that compared two types of probiotics (*L. acidophilus* and *B. longum*), neither improved intestinal permeability, but *B. longum* probiotic attenuated hepatic fat accumulation. However there are few human studies on the efficacy of probiotics in the prevention or treatment of NAFLD.

Hepatitis viruses, especially B and C, are known to cause long-term hepatocellular injury. As in other hepatic diseases, the plasma level of endotoxin increases in these patients because of changes in the gut microbiota [41]. Several studies evaluated the effects of probiotics in patients with hepatitis B virus (HBV) and hepatitis C virus (HCV). A research study achieved with *Bifidobacterium adolescentis* SPM0212 lead to increased expression of myxovirus (Mx) resistance A, an interferon (IFN)-inducible antiviral effector. Further, the extracellular surface

antigen of HBV level decreased depend by the dose up to 50% and gene expression was inhibited by 40% in hepatoma *cell* line HepG2.2.15 [43].

Also, in the literature have been reported studies regarding treatment with probiotic of patients with cirrhosis. Zhang et al. [44] used a cirrhotic-rat model with modified gut microbiota. In this research it was observed that the effects on total bilirubin (BT) and the ratio between aerobic and anaerobic bacteria were similar in healthy and cirrhotic rats. After administration of norfloxacin and probiotics to modify the gut microbiota, BT, liver function and endotoxemia were estimated. Cirrhotic rats showed a higher population of *Enterobacteriaceae* compared to healthy rats. It was concluded that treatment with bifidobacteria decreased the amount of *Enterobacteriaceae* and endotoxin level and increased the amount of *Lactobacillus* compared with healthy rats [44]. There are limited studies suggesting the role of probiotics for hepatocellular carcinoma. Chávez-Tapia et al. [41] reported in his article that clinical data the next probiotics—*L. rhamnosus LC705* and *Propionibacterium freudenreichii subsp. Shermanii,* maybe reduce the biologically effective dose of aflatoxin exposure. Similar data from murine models with *L. rhamnosus GG* were reported. Particularly after aflatoxin exposure, lower expression of c-myc, cyclin D1, bcl-2 and rasp-21/g3pdh were found [41].

In recent years also, several studies have shown that probiotics have beneficial effects and after liver transplantation. In a research by [45] patients who suffered for liver transplant were allocated to groups that received one of three treatments: live *L. plantarum* 299 strain and prebiotics (fibre), heat-killed lactobacilli and fibre, or selective bowel decontamination. Also, all patients received early enteral feeding. Patients who intake live lactobacilli and fibre developed a lower amount of bacterial infections (e.g. 13%) when compared to patients that underwent selective bowel decontamination: 48% [45]. However, probiotic bacteria such as *Saccharomyces cerevisiae* and *Lactobacillus* have been associated in some studies to the development of sepsis but the use of probiotics in patients who underwent a liver transplant requires at this point, a much more careful analysis of their safety [41].

2.2.4 Probiotics in urogenital and vaginal disease

According to the Centers for Disease Control and Prevention (CDCP), more than 1 billion women around the world suffer from non-sexually transmitted urogenital infections, such as bacterial vaginitis (BV), urinary tract infection (UTI) and several other yeast infections [46]. The dominant microflora in a healthy human vagina is comprised from a variety of *Lactobacillus* species with crucial role in protecting women from genital infections. A slight change in lactobacilli concentration can result in microbial disproportion in the vagina, causing a quantitative and qualitative modification from normally occurring lactobacilli to a mixed microflora controlled by anaerobic bacteria such as *Gardnerella vaginalis*, *Bacteroides* spp., *Prevotella* spp., and *Mobiluncus* species [47]. Commane et al. [48] reported in a research study the importance of probiotics in a woman's urogenital wellbeing. It was confirmed that by supplementing with probiotics (*L. rhamnosus* GR-1 and *Lactobacillus reuteri)* it can stimulate the colonisation of beneficial microbiota and may improve the vaginal health. Daily oral consumption of probiotics such as *L. rhamnosus* and *L. fermentum* exhibited the modification of the vaginal flora [48]. It is well-known that there is an association between abnormal vaginal microbial flora and an increased incidence of urinary tract infection (UTI). When administered twice daily orally the only strains clinically shown to have an effect are *L. rhamnosus* GR-1 and *L. reuteri*, these strains reduce recurrences of UTI and restored a normal lactobacilli dominated vaginal flora in patients [49]. The smallest imbalance in the microbial composition greatly influences the health of the vaginal microenvironment, potentially leading to compromised state

of BV and UTI. The primary solution for compromised state would be balancing the number of *Lactobacillus* spp. via the supplementation of probiotics [50].

2.2.5 Probiotics in cardiovascular diseases and lipid metabolism

Cholesterol is a precursor in many biochemical processes of the body and plays a vital role in many functions, like as production of steroidal hormones, while extreme cholesterol in the blood can lead to arterial clogging and increases the risk of heart disease and/or stroke. Patients with hypercholesterolemia showed the risk of heart attacks three times higher, compared to patients with normal blood lipid values [51]. The scientific literature reported some probiotic strains with hypocholerolemic effects, such as: *L. bulgaricus, L. reuteri, and B. coagulans*. Also, clinical research in humans with *L. acidophilus* L1 milk, revealed a significant reduction in serum cholesterol. Further, a clinical trial on 32 hypercholesterolemic patients that consumed low-fat yogurt with *B. longum* BL1 displayed a significant decline in triglycerides, total serum and LDL cholesterol. Also, HDL cholesterol was increased with 14.5% [52]. Thirty-two hyperc-holesterolemic men and women were intake *L. acidophilus* CHO-220 and inulin, during a randomised, double-blind, placebo-controlled, and parallel-designed trial. This research study demonstrated that plasma total cholesterol and low-density lipoprotein (LDL)-cholesterol reduced by 7.84 and 9.27%, respectively, after 12 weeks [53]. Worldwide, it is known that coronary heart disease (CHD) is one of the major causes of adult's death. The main coronary arteries supplying the heart are no longer able to provide sufficient blood and oxygen to the myocardium, mainly because of the accumulation of plaques in the intimae of arteries [54]. Ranjbar et al. [54] concluded that in recent years, several foods enriched with probiotics were produced industrially. These foods have recently been subject to more research for their beneficial effects on the gut microflora and links to their systemic effects on the lowering of lipids known to be risk factors for CHD.

2.2.6 Probiotics in oral health

The human mouth harbours diverse microbiomes in the human body such as viruses, fungi, protozoa, archaea and bacteria and they cause different diseases. From a dental point of view the bacteria cause two common diseases: dental caries and the periodontal (gum) diseases. The most used probiotics for oral health are species of *Lactobacillus* and *Bifidobacterium*. In a double-blind, placebo-controlled trial the consumption of *Streptococcus salivarius* K12 decreased the occurrence of plaque and also, reduced the concentration of *Streptococcus mutans* [55]. It is known that *Streptococcus uberis* and *S. oralis* also can inhibit the periodontal pathogens [56]. Additionally, the halitosis and the volatile sulphur compounds synthesis could be prevented by probiotics consumption.

Bowen [57] declared that the evidence for periodontitis is less than dental caries, but the use of probiotics to manage the oral microflora appears tobe an effective method to control oral conditions [57]. Many more studies are needed to understand the mechanism by which these probiotics colonise and affect the oral cavity. Is needed to better understand how they improve oral health.

2.2.7 Probiotics in lactose intolerance

Daliria and Lee [58] supposed that lactose is an important nutrient in all mammalian neonates, almost all of them have the capability to metabolise lactose to glucose and galactose. It is known that in humans, lactase activity decreases during mid-childhood [58]. Medical research reports that lactose intolerance is determined

by blood glucose concentrations, and breath hydrogen test following ingestion of a lactose load [58] and symptoms include: abdominal distress like diarrhoea, bloating, abdominal pain and flatulence. The researchers noticed that treatment with probiotics (such as *Lactobacillus bulgaricus* and *Streptococcus thermophiles*) relieves symptoms of lactose intolerance. It is also observed that consumption of milk containing *Bifidobacterium longum* and *L. acidophilus* cause significantly less hydrogen production and flatulence. In researches where was used a combination of *Lactobacillus casei shirota* and *Bifidobacterium breve* Yakult has shown better effect on patients and improved the symptoms of lactose intolerance significantly [59].

2.2.8 Probiotics in cancer

Kerry et al. [50] declared that as per World Health Organisation (WHO) cancer fact sheet this is a dreadful disease affecting peoples all over the globe. Approximately 14 million new cases and 8.2 million cancer-related deaths added till 2012. The global cancer deaths are from Asian, African, and American continents (more than 70%) [60]. *In vitro* studies, probiotic strains, *Lactobacillus fermentum* NCIMB-5221 and -8829, revealed the highly potential in destroying the colorectal cancer cells and promoting normal epithelial colon cell growth by producing the SCFAs (ferulic acids). This probiotics were compared to other probiotics (*L. acidophilus* ATCC 314 and *L. rhamnosus* ATCC 51303) known with tumorigenic properties [61]. Also, *L. acidophilus* is known to prolong the induction of colon tumours. It was demonstrated that feeding milk and colostrum fermented with *L. acidophilus* resulted in 16–41% reduction in tumour proliferation [62]. Also, the other probiotic *L. bulgaricus* has also been reported to induce antitumor activity against sarcoma-180 and solid Ehrlich ascites tumours [63]. Probiotics could play a significant role in neutralising cancer but research is limited only to *in vitro* tests.

3. Prebiotics

Like probiotics, prebiotics is also being widely explored for their utility in the various field of applied science, more specifically as nutrients and supplements [50]. Food and Agriculture Organisation (FAO)/WHO defines prebiotics as a nonviable food component that confer health benefit(s) on the host associated with modulation of the microbiota [62].

Sources of prebiotics are as follows: breast milk, soybeans, inulin from diverse sources (Jerusalem artichoke, chicory roots), raw oat, wheat bran, barley bran, yacon roots, non-digestible carbohydrates (non-digestible oligosaccharides). From prebiotics, only bifidogenic, non-digestible oligosaccharides, especially inulin, and its hydrolysis products, such as oligofructose, and (trans) galactooligosaccharides (GOS), achieve all the criteria for prebiotics term [64]. Prebiotics can be obtained naturally from sources like vegetables, fruits, and grains consumed in our daily life but are also artificially prebiotic products such as: lactulose, galactooligosaccharides, fructooligosaccharides.

Kuo [65] reported that an ideal prebiotic should be:

- resistant to the actions of acids in the stomach, bile salts and other hydrolysing enzymes in the intestine;
- not be absorbed in the upper gastrointestinal tract;
- be easily fermentable by the beneficial intestinal microflora.

Prebiotics not only serve as an energy source because their presence of prebiotics in the diet may lead to numerous health benefits. Several health benefits are reported in scientific literature, such as inhibition of the development of pathogens, reducing the prevalence and duration of diarrhoea, increases the absorption of minerals, mostly of magnesium and calcium, exerting protective effects to prevent colon cancer and providing relief from inflammation and other symptoms associated with intestinal bowel disorders.

Several studies demonstrated that the colorectal carcinoma was less present at people who consume a lot of vegetables and fruits. The inulin and oligofructose from fruits and vegetables could suppress the disease [66]. When it comes to the advantages of prebiotics, it can be mention the reduction of the blood LDL (low-density lipoprotein) level, stimulation of the immunological system, increased the calcium absorbability, preservation of adequate intestinal pH value, low caloric value, and alleviation of symptoms of peptic ulcers and vaginal mycosis [67]. Other benefits of inulin and oligofructose on human health could be the prevention of carcinogenesis, as well as the support of lactose intolerance or dental caries treatment [68]. Also, prebiotics are useful in combating pathogenic microorganisms, such as *Salmonella enteritidis* and *Escherichia coli*, and reduce odour compounds and [69] confirmed a positive effect of fructooligosaccharides (FOS) on protection against *Salmonella typhimurium* and *Listeria monocytogenes* infections. Pokusaeva et al. [70] said that prebiotics are also implicated in enhancing the bioavailability and uptake of minerals, lowering of some risk factors for cardiovascular disease, and promoting satiety and weight loss.

Prebiotics have been reported to play a beneficial role in controlling the IBD. A major reduction in the number of bacteriodetes in faeces was reported in patients with chronic pouchitis treated with 24 g per day of inulin [71]. In another study, 10 Crohn's Disease patients receiving 15 g of FOS demonstrated a reduced disease activity index [72]. In another randomised study involving 103 Crohn's Disease patients who received FOS 15 g/day these showed no clinical improvement however, though no change in IL-12 was observed it was able to reduce IL-6 of lamina propria dendritic cells [1].

Kerry et al. [50] suggests that even with their enormous nutritional and medicinal benefits, research concerning screening new versatile prebiotics is quite deficient. Therefore, the research should be focused on identifying new healthy supplements, while screening novel prebiotic strains should be a major concern.

4. Synbiotics

Due to the expansion of microbial research were discovered synbiotics as a combination of probiotics and prebiotics products which provide the survival and the implantation of the live microorganism dietary supplements in the gut [73]. The synergistic welfares are more proficiently promoted when both the probiotic and prebiotic act together in the living system. It is known that the symbiotic association between prebiotics and probiotics significantly improve the human health [50]. From the medical point of view the term of synbiotic product positively influence the host through improving the survival and implantation of live microbial dietary supplements in to the gastrointestinal tract and stimulating the growth and/or activating the metabolism of health promoting bacteria [62]. Since the word synbiotics suggests synergism, this term should be reserved for products in which the prebiotic compound(s) positively influence the probiotic organism(s) [74]. Markowiak and Śliżewska [1] suggests that when develop a synbiotic product, the most important aspect that have taken into account, is the selection of an appropriate probiotic and

prebiotic, that can act separately on the host's health. The prebiotic compounds should selectively stimulate the growth of probiotics, with beneficial effect on human health and not to be able to stimulate the other microorganisms.

Lactobacillus spp., *Bifidobacteria* spp., *S. boulardii*, *B. coagulans* are one of the probiotic strains that are used in synbiotic formulations, whereas the prebiotics used are as follows: oligosaccharides (fructooligosaccharide (FOS), GOS and xylo-seoligosaccharide (XOS)), and inulin (from natural sources like chicory and yacon roots) [62]. Synbiotics consumption by humans includes the following beneficial effects:

- Increased levels of lactobacilli and bifidobacteria and balanced gut microbiota.
- Prevention of bacterial translocation and reduced incidences of nosocomial infections in surgical patients.
- Improvement of liver function in cirrhotic patients.
- Improvement of immunomodulating ability [75].

In adult subjects with non-alcoholic steatohepatisis (NASH) in a randomised study what used of a synbiotic product which contained five probiotics namely: *Lactobacillus plantarum*, *L. delbrueckii* spp. *bulgaricus*, *L. acidophilus*, *L. rhamnosus*, *Bifidobacterium bifidum* and inulin as a prebiotic has been demonstrated a significant reduction of intrahepatic triacylglycerol (IHTG) within 6 months [1]. Fifty-two adults participated for 28 weeks in a research trial based on the effects of the synbiotic product. The synbiotic comprised a mix of probiotic strains: *Lactobacillus casei, L. rhamnosus, Streptococcus thermophilus, Bifidobacterium breve, L. acidophilus, B. longum, L. bulgaricus* and fructooligosccharides, as prebiotic. The authors stated that consumption of the synbiotic product resulted in the inhibition of nuclear factor-kB (NF-kB) and a condensed production of tumour necrosis factor α (TNF-α) [76].

Moreover, synbiotics seems to be quite attractive for improving the immune system. A significant decrease in the levels of C-reactive protein and also increase the glutathione levels was obtained through combination of *B. coagulans* with inulin, in diet for 6 weeks [77].

Recently, commercial interest in functional foods based on synbiotics has improved due to the awareness of the welfares for gut health, disease prevention and therapy. Investigates in this scientific zone is presently concentrated on designing new functional foods, as well as on screening new strains with capability to inhabit the human gut, along with their aptitude to metabolise new prebiotics [50]. Trials and investigation *in vitro* and *in vivo* demonstrated that the beneficial effects of using probiotics, prebiotics, and synbiotics in health are much more active than their unitary use known till present. Nevertheless, more investigates concerning the designing new mixtures of probiotics, prebiotics and synbiotics are imperative necessary for achieve further opportunities of improving nutritional and clinical health.

5. Conclusion

The use of probiotics, prebiotics, and synbiotics in health is emerging as a promising therapy which is generally safe in different disease. Probiotics, probiotics and synbiotics have systemic effects on the urogenital disease, liver disease, oral health and immune system. There are many published reports on the use of probiotics in

humans but information on prebiotics and synbiotics is quite a few. It seems that we will see and in the coming years further studies on combinations of probiotics and prebiotics, and further development of synbiotics. It is possible that future studies may explain the mechanisms of actions of those components, which may confer a beneficial effect on human health. However, the health claims made needs to be substantiated and firmly established by properly designed large scale clinical trials on human body. Therefore, current focus is on evaluating new strains of probiotics, a new prebiotics and new synbiotics products and their applicability in biomedical/clinical research, paving a new direction for exploration and exploitation of probiotics, prebiotics and synbiotics aimed at improving human health. There is a need for more randomised, placebo-controlled clinical trials with adequate statistical power. I encourage researchers to submit possible publications in peer-reviewed journals of all clinical trials, whether the outcome is positive, negative or adverse, because the scientific and medical world needs it relevant information on the dose–response effects, efficacy, and safety of probiotic, prebiotic and synbiotic products. At present, the available information on current probiotics, prebiotics and synbiotics provides convincing safety records. I believe it is highly likely that in the near future, the vast amount of research on the beneficial impact of the probiotics, prebiotics and synbiotics on human wellbeing will suppose discovery and development of innovative products derived from our microbiota. Further, these may belong to uncommon and formerly uncharacterized microorganisms with rare assets, or perhaps could be microorganisms formerly known as pathogens or pathobionts. These progresses will represent new trends but also significant challenges for scientific and medical research, for industrial exploitation and for human health and clinical nutrition.

Conflict of interest

Author declares no conflict of interest.

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Chapter 5

African Fermented Food Condiments: Microbiology Impacts on Their Nutritional Values

Nurudeen Ayoade Olasupo and Princewill Chimezie Okorie

Abstract

Fermented food flavoring condiments are products usually derived from the fermentative activities of microorganisms on vegetable proteins of legumes or oil seeds. Africa is a continent that is endowed with many fermented food condiments. These condiments, apart from their flavoring properties, serve as a cheap source of plant protein to the populace, especially the rural dweller whose staple foods are mainly carbohydrate based. The production dynamics of these condiments vary from country to country. However, the microbial interplay during their production and their nutritional qualities appear to be same. This chapter seeks to evaluate the range of substrates employed in the production of fermented condiments of African origin, the microbial interplay in their production and their nutritional values.

Keywords: microbiology, nutrition, fermentation, African fermented condiments

1. Introduction

Fermented foods constitute a significant component of African diets. There are many fermented foods known in Africa. These foods are classified into five major categories based on the substrate from which they are derived [1] and they include fermented food condiments among others.

Condiment is defined as a spice, sauce or other food preparation that is added to food to impart a particular flavor or enhance its taste (example salt). Fermented food flavoring condiments are products usually derived from the fermentative activities of microorganisms on vegetable proteins of legumes or oil seeds origin [2, 3]. They include *iru* from Africa locust bean, *ugba* from African oil bean seed and *ogiri* from melon seeds among others. These fermented food condiments are known to be good sources of proteins and vitamins [1, 4].

The use of fermented vegetable proteins as seasonings is wide spread in Africa, especially among the rural dwellers. In West Africa, some of the common fermented vegetable condiments include *iru or dawadawa* from locust bean (*Parkia biglobosa*) (**Figure 1**), *ogiri* from melon seeds (*Citrullus vulgaris*) (**Figure 2**), *daddawa* from soybean (*Glycine max*), *soumbala* from soybean (*Glycine max*) (**Figure 3**), *ugba* from African oil bean seed (*Pentaclethra macrophylla*) (**Figure 4**) and *owoh* from

Figure 1.
Unfermented seeds of African locust bean (a) and fermented seeds of African locust bean (b) (iru/ dawadawa/ Afitin/Sonru/soumbala). Source: [31].

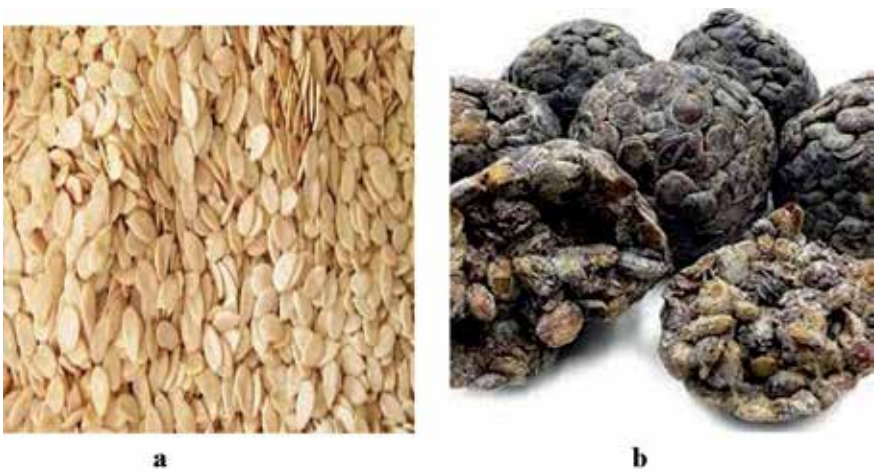

Figure 2.
Unfermented melon seeds (a) and fermented melon seeds (b) (ogiri). Source: [22].

Figure 3.
Soumbala (in balls) and the seeds used for their preparation. Source: [11].

Figure 4.
African oil bean seeds (a) and fermented slices of the oil bean cotyledon (b) ugba. Source: [57].

cotton seeds (*Gossypium hirsutum*). **Table 1** presents a comprehensive list of fermented food condiments of African origin.

These fermented condiments bear different names according to the country or region of the continent from which they are produced. African locust bean tree (*Parkia biglobosa*), for instance, is one of the most common plants whose seeds are used as protein source condiment after fermentation. It is consumed by various socioethnic groups in the West African subregion, and it bears different names across the region. It is popularly known as *afitin/sonru/iru* in Benin [5–7], *iru/dawadawa* in Nigeria [8, 9], *soumbala* in Burkina Faso [10, 11] and *netetu* in Senegal [12].

Raw material	Product	Country	Reference
Soy bean	*Dawadawa*	Nigeria	[19]
	Soumbala	Burkina Faso	[11]
	Tempeh	Ghana	[19]
Melon seed	*Ogiri*	Nigeria	[23]
Castor oil seed	*Ogiri igbo*	Nigeria	[26]
Fluted pumpkin seed	*Ogiri ugu*	Nigeria	[27]
African locust bean	*Dawadawa/iru*	Nigeria	[17]
	Afitin/Sonru	Benin	[5]
	Soumbala	Burkina Faso	[10]
	Netetu	Senegal	[12]
African oil bean seed	*Ugba*	Nigeria	[38]
African yam bean	*Ogiri*	Nigeria	[38]
Cotton seed	*Owoh*	Nigeria	[95]
Bambara groundnut	*Ogiri okpei*	Nigeria	[31]
Prosopis africana seed	*Okpehe*	Nigeria	[28]
Roselle plant	*Bikalga*	Burkina Faso	[14]
	Dawadawa botso	Niger	[14]
	Furundu	Sudan	[14]
	Mbuja	Cameroon	[14]
Fish	*Lanhouin*	Benin	[15]

Table 1.
Common fermented food condiments of African origin.

The Roselle plant (*Hibiscus sabdariffa* L.) is another herbal shrub whose seeds are rich in protein, oil and dietary fiber [13]. The seeds of this plant are widely used in alkaline fermentation for the production of food condiment popularly known as *bikalga* (Burkina Faso), *dawadawa botso* (Niger), *datou* (Mali), *furundu* (Sudan) and *mbuja* (Cameroon) [14].

Even within a country, the names of these condiments vary from one part to another. The origin of such names, however, could be attributed to a number of factors which include (a) the region or area of manufacture of the condiment, (b) the type of legume or oil seed used and (c) the spelling according to the region or area. In Nigeria, for instance, the Yorubas of the Southwestern Nigeria locally call fermented condiments *iru*, the Hausas of the Northern part call it *dawadawa* and the Ibos of the Eastern part call it *ogiri* [1]. *Owoh*, on the other hand, is a popular name for fermented condiments among the Urhobos and Itsekiris in the Niger Delta region, while the Igala and Idoma people of the Middle Belt region call it *okpiye* [3].

The conventional substrates for these condiments production are diverse but are mainly legumes and oil seeds. *Lanhouin* is, however, a fish-based condiment, which is common in Benin [15]. *Lanhouin* is used as a taste- and flavor-enhancing condiment in some main dishes such as vegetable, slimy vegetable and tomato sauces. One condiment can be produced from more than one raw material. For instance, in Nigeria, *dawadawa* and *iru* are locally produced from three materials: African locust bean (*Parkia biglobosa*), soybean (*Glycine max*) or Bambara groundnut (*Vigna subterranea*) [16–21]. *Ogiri* is traditionally prepared by fermenting melon seeds (*Citrullus vulgaris*) and fluted pumpkin (*Telfairia occidentalis*) or castor oil seed *(Ricinus communis*) [22–27]. *Owoh* is produced from fermented seeds of the cotton plant (*Gossypium hirsutum*) or African yam bean (*Sphenostylis stenocarpa*)

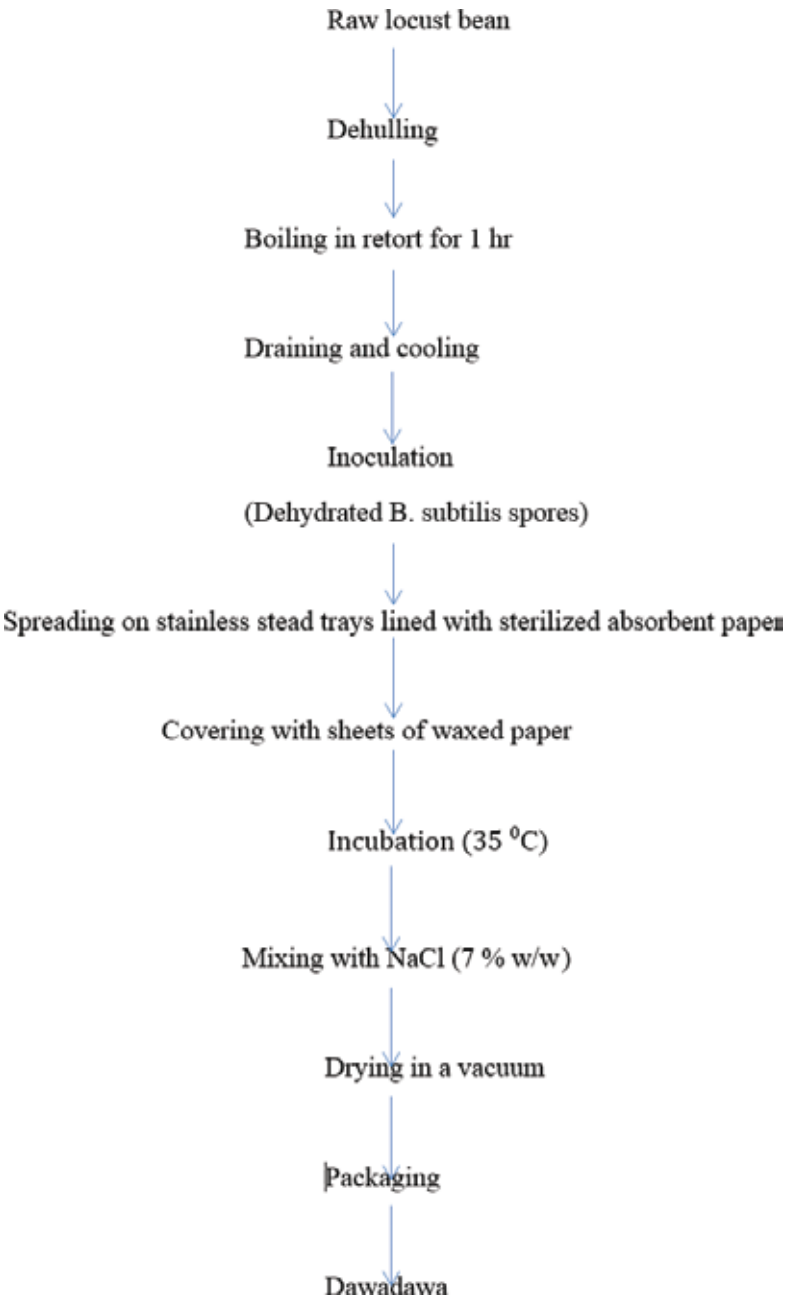

Figure 5.
Flowchart for the preparation of dawadawa. Source: [31].

[28–30]. On the other hand, *okpiye* is prepared from the seeds of *Prosopis africana* [31–33]. Almost any edible plant material can be subjected to fermentation to produce condiment.

Fermented food condiments play very important role in the diet of many Africans. They are used to enhance the flavor of many dishes including soups and sauces [6, 34]. These fermented food condiments are also known to be good sources of protein and vitamins [1, 4]. Apart from the flavoring attributes, they contribute to the protein intake of the consumers. The significance of this fact is better appreciated when you realize that most of the meals in many parts of West, Central, and

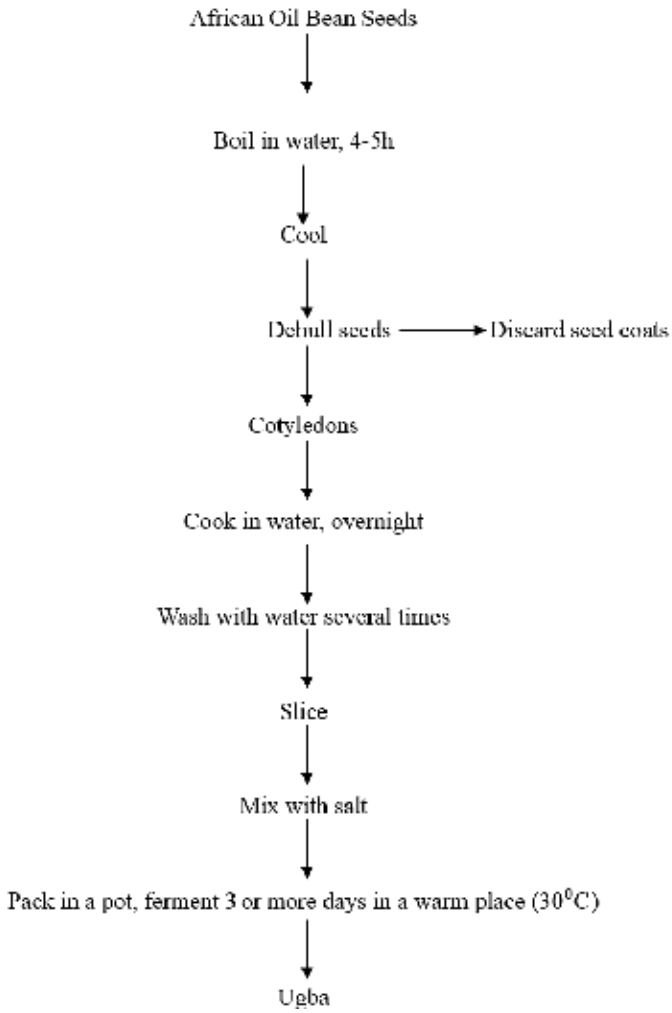

Figure 6.
Flowchart for the preparation of ugba. Source: [97].

Southern Africa are made of starchy roots and grains and have to be taken with soups to which these condiments are an essential input [3].

The traditional methods of preparation of these condiments are generally very laborious, time and energy consuming and are usually carried out with rudimentary utensils. The essential steps in the preparation of these condiments are similar with minor differences occurring from one condiment to another and among different localities [30]. In Benin Republic, for instance, *ikpiru* and *yanyanku* are two additives used for traditional alkaline fermentation of African locust bean (*Parkia biglobosa*) to obtain the popular *afitin/sonru/iru* condiment [35]. These additives are, however, not involved in the production of the same condiment in the other neighboring countries. The basic steps in the production of these condiments involve shelling/decorticating and dehulling of the seeds, the seeds are washed and wrapped in several layers of leaves and left to ferment. In some other methods, the seeds are spread in calabashes that are stacked together and wrapped in several jute bags and left to ferment. These conditions create low oxygen tension and help to maintain the optimum conditions of temperature and humidity necessary for the fermentation process. The fermentation time varies from one product to another

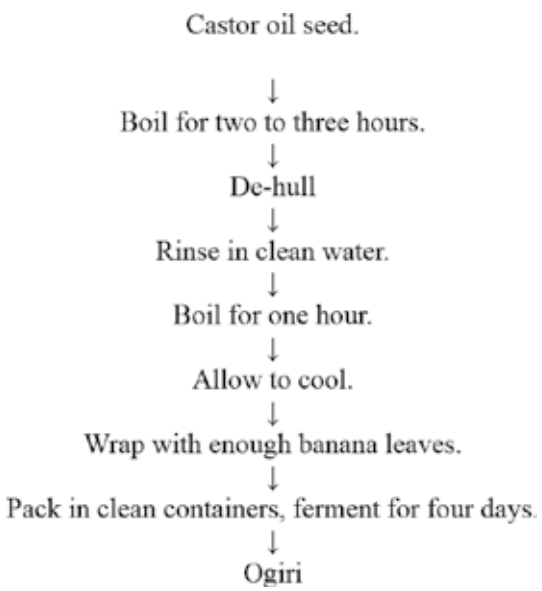

Figure 7.
Flowchart for the preparation of ogiri. Source: [98].

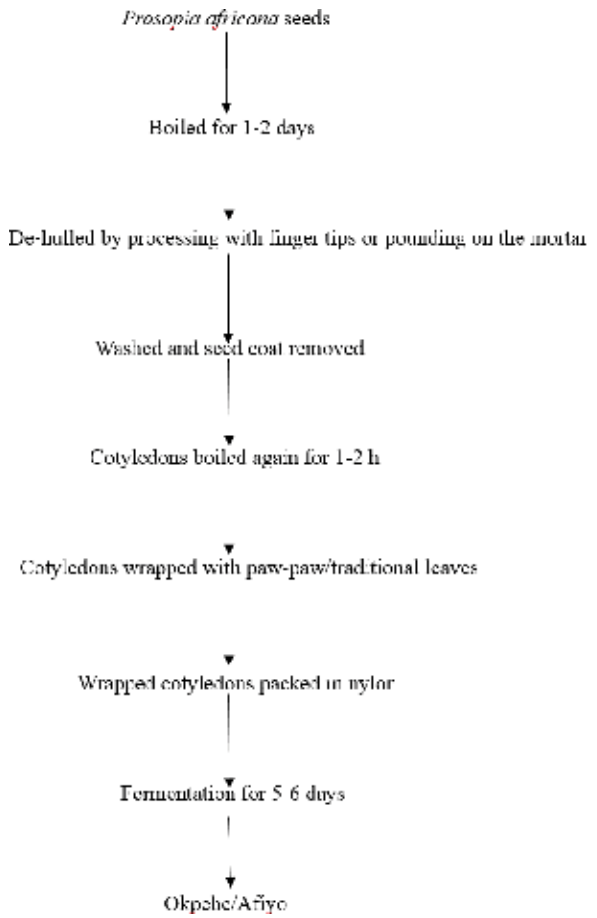

Figure 8.
Flowchart for the preparation of okpehe. Source: [43].

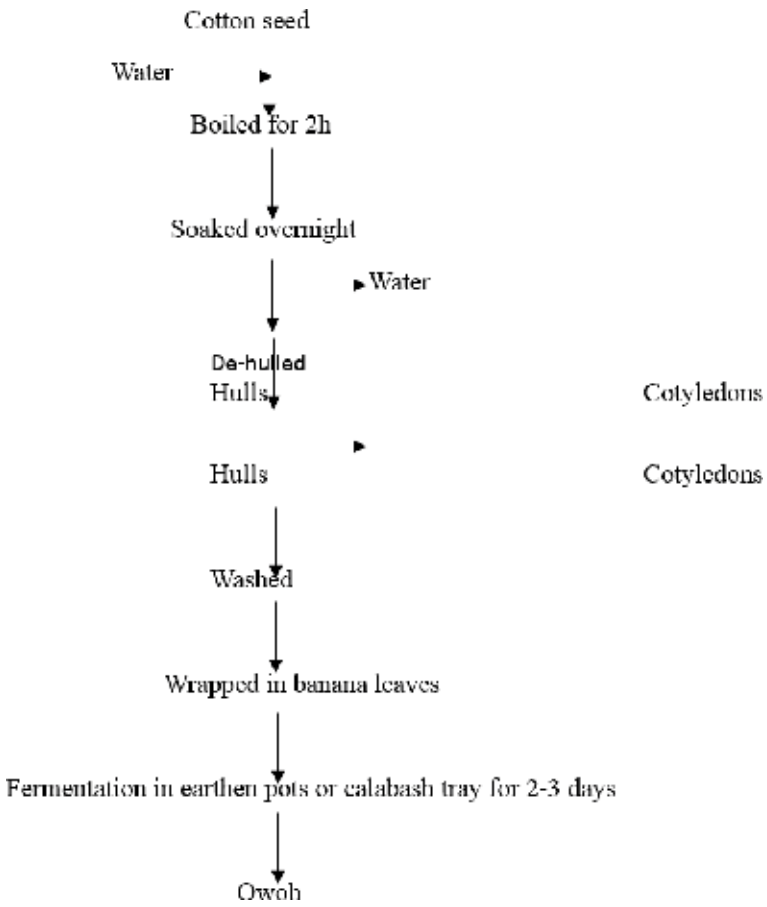

Figure 9.
Flowchart for the preparation of owoh. Source: [28].

and from one processor to another. Generally, it ranges from 48 to 120 h (2–5 days). **Figures 5–9** show the flowcharts for the fermentation of African locust bean seeds, African oil bean seeds, castor oil seeds, *Prosopis africana* seeds and cotton seeds, respectively, into various food condiments.

2. Microbiology of African fermented condiments

The microbiota in any fermenting food matrix is a function of the hygienic status of the production environment, the utensil and the raw material used and the handlers. The traditional fermentation method employed in the processing of most fermented African condiments is by chance inoculation [2, 30, 36]. The microbial interaction during their production is, therefore, determined by the microbiological status of the raw material, utensils, handlers and production environment. These factors vary from one community to the other and from one processor to another. The microbial interplay in the fermenting mash, therefore, may also vary from one processing community to the other and from one processor to another and even from one batch of production to another (**Table 2**). During fermentation of these condiments, the microorganisms use the nutritional components of the substrates, converting them into products that contribute to the chemical composition and taste of the final product [30, 37].

Food	Area of production/ consumption	Raw material	Microorganisms
Dawadawa or *iru*	Most of West Africa especially northern African parts	African locust bean (*Parkia biglobosa*) Soybean (*Glycine max.*)	*Bacillus subtilis* *B. licheniformis*
Ogiri	Southwestern Nigeria	Melon (*Citrullus vulgaris*)	*Bacillus* sp.(predominant), *Proteus*, *Pediococcus*
Ogiri-nwan	Southwestern Nigeria	Fluted pumpkin bean (*Telfairia occidentalis*)	*Bacillus* sp. (proteolytic)
Ogiri-igbo (ogiri-agbor)	Southeastern Nigeria	Castor oil seed (*Ricinus communis*)	Various *Bacillus* species: *B. subtilis, B. megaterium, B. firmus*
Ogiri-saro (*sigda*)	Sierra Leone, Sudan	Sesame seed (*Sesamum indicum*)	*Bacillus* sp.
Ogiri-okpei/Okpehe	Middle belt Nigeria	Mesquite (*Prosopis africana*)	*Bacillus* sp.
Ugba (apara)	Eastern Nigeria	African oil bean (*Pentaclethra macrophylla*)	*Bacillus subtilis* *Micrococcus* sp.
Owoh	Midwestern Nigeria	Cotton seeds (*Gossypium hirsutum*)	*Bacillus* sp.
Bakalga	Niger, Mali, Sudan, Burkina Faso	Kartade red sorrel (*Hibiscus sabdariffa*)	*Bacillus subtilis*

Source: [3].

Table 2.
Some important fermented vegetable foods of Africa and their fermenting organisms.

The major fermenting microorganisms involved in the fermentation process of most vegetable protein (fermented condiments) have been identified as proteolytic *Bacillus* species, e.g., *B. subtilis, B. megaterium, B. circulans* [2, 30, 33, 38]. *Bacillus subtilis*, however, appears to be the most predominant of all the *Bacillus* species. The endospores of these bacilli are believed to be associated with the cotyledons of these seeds from the onset of the fermentation process.

Proteolysis is the major biochemical activity taking place during the fermentation of most fermented food condiments that are of plant origin [39, 40]. Proteolytic activity has been found to steadily increase with increase in the fermentation period during the production of these food condiments [39, 41]. Due to the high level of hydrolytic enzyme production by *Bacillus* species, all the species have been reported to have one or more enzymatic hydrolytic properties during legume fermentation [42, 43]. However, it appears that *Bacillus subtilis* is the most adapted and dominant species. *Bacillus subtilis* produces high levels of protease, amylase and polyglutamic acid (responsible for mucilage production that is common in fermented vegetable protein) [43].

Protein has been identified as one of the major components of the legumes and oil bean seeds used for the fermentation of these condiments [38]. Metabolic and enzymatic hydrolytic activities of the *Bacillus* species serve to break down the protein into amino acids [39, 40, 43–46]. An increase in the population of *Bacillus* species from the beginning of the fermentation process till the end had been reported [41]. Microorganisms belonging to other groups of bacteria are also associated with the fermentation of these condiments. They include species of *Escherichia, Proteus, Pediococcus, Micrococcus, Staphylococcus, Streptococcus, Alcaligenes, Pseudomonas, Corynebacterium and Enterococcus* [17, 18, 20, 37, 41, 47–49]. *Staphylococcus* and

Micrococcus species are very active at the early stage of the fermentation process. They multiply rapidly within 24 h of fermentation and then decrease as fermentation progresses [41]. Their role in the fermentation process is, however, lower compared to that played by the *Bacillus* species. Species of *Escherichia*, *Proteus* and *Pediococcus* generally play a minor role in the fermentation process [38, 50, 51].

Besides proteolysis, other biochemical changes mediated by microorganisms during the production of these condiments include production of flavor-enhancing compounds, production of vitamins and essential fatty acids and degradation of indigestible oligosaccharides responsible for flatus factors [45]. A significant increase in vitamins, such as thiamine and riboflavin, has been observed in these condiments, which is possibly due to riboflavin synthase associated with the *Bacillus subtilis* [45]. A reduction in the content of flatus factors [stachyose, raffinose and melibiose] in fermented condiments of African origin has been reported [52]. The reduction is as a result of sucrase activities of the *Bacillus* group and possibly by the α-galactosidase activities of other microorganisms in the fermenting mash [39, 53].

Members of the *Enterobacteriaceae* have also been associated with the ecology of fermenting plant protein especially at the early stages of production [31, 54]. These species do not survive until the end of the fermentation, presumably because of the modified environment [41]. It is evident that production of these fermented condiments is initially mediated by a diverse microbial flora, which eventually becomes Gram-positive flora (a reflection of many African fermented foods) [26].

The identification of these organisms have been based on phenotypic approach with its inherent shortcomings, especially its inability to isolate and identify viable, but unculturable, microorganisms. Unculturable, yet viable, microorganisms are known to be in most food matrix [55, 56]. In a recent study [57] on the processing methods and safety of a fermented food condiment in Nigeria (*ugba*), the author deployed both phenotypic and molecular tools in his study. New bacterial species of *Arthrobacter, Empedobacter, Providencia, Brevibacterium, Elizabethkingia, Acinetobacter, Burkholderiales, Proteobacterium, Wautersiella, Dysgomonas, Zymomonas* and *Flavobacterium* were uniquely identified by the clone library technique employed. The study, therefore, underscores the need to deploy molecular techniques in the evaluation of the microbiology of these African fermented food condiments. It is possible that the microbial structure reported for these products could be wider than is currently recorded.

3. Nutritional properties

Fermentation has generally been observed to improve the nutritional qualities and safety of fermented food products [58–63]. Proximate analyses of most fermented vegetable protein of African origin have shown that these condiments are rich sources of protein, essential amino acids, vitamins and minerals. These components have been found to increase during the fermentation of these condiments [4, 63–65].

The substrates for the fermentation of these condiments harbor diverse microorganisms from the environment [66–68]. These microorganisms transform the chemical constituents of the raw materials during fermentation. The transformation has the following advantages: [i] enhance nutritive value of the products; [ii] enrich bland diets with improved flavor and texture; [iii] preserve perishable foods; [iv] fortify products with essential amino acids, health promoting bioactive compounds, vitamins and minerals; [v] degrade undesirable compounds and antinutritional factors; [vi] impart antioxidant and antimicrobial properties; [vii] improve digestibility and [viii] stimulate probiotic functions. Fermentation of these

products also results in a lower proportion of dry matter in the food products, and the concentration of the vitamins, minerals and protein appears to increase when measured on dry weight basis [4, 63–65, 69, 70].

A large percentage of Africa's population live below poverty line with diets that are poor in protein and other essential nutrients [3, 71]. Fermented food condiments have been found to be rich in proteins and other essential nutrients and, therefore, serve as supplements for these nutrients outside their usage as flavoring agents [72–75] (**Table 3**). *Bikalga*, for instance, is a popular fermented food condiment in Benin Republic, which is considered as an excellent source of protein with essential amino acids. It also contains lipids, carbohydrates, essential fatty acids and vitamins [11, 76]. Many families often use *Bikalga* as a meat substitute. Most African fermented food condiments are used to improve nutritional values of foods as well as their sensory properties and as taste enhancer [70].

Generally, a significant increase in the soluble fraction of amino nitrogen of a food is observed during fermentation [77]. Investigation by Niba [78] showed that protein quality in grain cereals is improved during fermentation due to depletion of trypsin inhibitors, which increases the digestibility of various amino acids.

Fermentation markedly improves the digestibility, nutritive value and flavor of raw seeds [79–81]. Studies on the effect of fermentation on the nutrient content of some unfermented leguminous seeds (locust beans and oil bean seeds) showed that protein and fat increased when fermented, whereas the quantity of carbohydrates decreased [82]. Increased levels of the amino acids were also reported except for arginine, leucine and phenylalanine. Similar results have been reported for other seed legumes [26, 52]. The organisms involved in the fermentation processes, especially *Bacillus* sp., produce proteolytic enzymes, which hydrolyze proteins to amino acids and peptides [18, 23, 26, 50, 83–85]. *Bacillus* strains obtained from fermenting African oil bean seed and locust beans have been found to produce glutamic acid and extracellular proteinases, which play active role in the fermentation process of these seeds [42, 86].

The proximate composition of some fermented vegetable protein (FVP) and their raw materials indicate that the major components are protein and fat (**Table 3**). The most significant reaction/change in the fermentation of proteins is their hydrolysis to free amino acids and other soluble nitrogen compounds. The amino acids produced vary, depending on the type of seed [fermenting substrate]. The peptides and amino acids are important in the evolution of the flavor of the condiments. Glutamic acid, an important flavoring component, has been observed in the fermentation of *ugba, iru* and *dawadawa* [87].

The major component of the carbohydrate content of legumes is starch, raffinose, melibiose and stachyose [26, 50]. During fermentation, these oligosaccharides

Proximate composition (%)						
Condiments	**Moisture**	**Ash**	**Crude fiber**	**Crude protein**	**Carbohydrate**	**Fat**
Iru/Dawadawa	52.0 ± 5.0	3.6 ± 0.1	4.0 ± 0.1	32.9 ± 0.1	16.3 ± 0.8	24.2 ± 0.1
Ogiri	44.1 ± 0.8	3.0 ± 0.0	15.6 ± 0.4	19.9 ± 0.8	25.2 ± 1.2	—
Owoh	46.6	2.21	6.01	16.37	14.06	20.76
Ugba	34.4	1.11	2.93	7.13	17.48	19.72
Okpehe	9.46	4.84	2.99	36.88	47.18	11.35
Source: Adapted from [4, 64, 99].						

Table 3.
Proximate composition of some African fermented condiments.

Mineral composition (mg/100 g)								
Condiments	**P**	**K**	**Na**	**Ca**	**Mg**	**Zn**	**Fe**	**Mn**
Iru	80.00	205.00	—	9.01	35.00	—	3.31	—
Ogiri	91.17	1075.00	369.36	78.60	58.72	1.17	14.50	1.15
Owoh	—	464.50	416.50	246.0	150.0	119.7	16.0	—
Ugba	291.02	110.39	172.06	208.92	334.98	9.23	42.46	26.87
Okpehe	—	183.1	—	45.3	—	14.2	10.2	4.2
Source: Adapted from [4, 64, 99].								

Table 4.
Mineral composition of some African fermented condiments.

are hydrolyzed to simple digestible sugars [88]. Assay of the fermenting mash of African oil bean seed and African locust bean showed activities of α- and β-galactosidases and sucrase [89], with α- and β-galactosidases being the highest. Other enzymes present are galactanase, glucosidases and fructofuranosidases and polygalacturonases. These enzymes are produced by *Bacillus* species, *Staphylococcus* species and lactic acid bacteria, the latter group producing α-galactosidase, and they play very active role in the hydrolysis of these oligosaccharides. The nutritional significance of hydrolysis of oligosaccharides is evident in the drastic reduction of the level of indigestible carbohydrates, which cause flatulence [89].

Oil constitutes a major component of the legumes and oil seeds, but lipolytic activities are minimal during the production of most African fermented food condiments. Low lipolytic activities were detected during *ugba* and *dawadawa* production. The lipolytic activities are attributed to *Staphylococcus* species in the fermentation medium [39, 90]. During fermentation, the free fatty acid fractions [FFA] are reduced from 0.6 to 0.1% w/w in the fermented seeds. No significant differences were observed between the fatty acid content of the raw seeds and the fermented seeds; the major components are palmitic acid, stearic acid, oleic acid and linoleic acid [91].

Many reports confirm that vitamin levels are higher in fermented vegetable protein foods than in the raw materials, especially for riboflavin, thiamine, niacin, vitamin C and folic acid [1, 89]. Food condiments made from vegetable proteins may be a good source of certain B vitamins, but they are found to be deficient in ascorbate and some fat-soluble vitamins, which are lost during fermentation. Fermentation significantly increases the content of thiamine, riboflavin and niacin in the African oil bean [92]. Similar changes were observed during the fermentation of melon seed and fluted pumpkin seed [93, 94].

Calcium, phosphorus and potassium have been observed to increase when African oil bean seed and African yam bean were fermented for condiment production [95, 96]. Similar observation has been made on other fermented condiments (**Table 4**). It is evident that most fermented food condiments of African origin are good sources of essential nutrients and could be used to produce complementary food supplements and macronutrients in fermented legumes and therefore enhance food quality. However, issues of quality inconsistency, poor keeping quality and safety observed with these products must be addressed.

4. Conclusion

Fermented condiments constitute an important part of diet of most Africans. These condiments, apart from their flavoring properties, serve as cheap source

of protein and other essential micronutrients to the consumers. The production process of most of these condiments is still based on spontaneous fermentation process with its inherent shortcomings. There is need, therefore, for more microbiological studies of their production process with the aim of establishing standardized protocols for their production.

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Chapter 6

Pursuing the Perfect Performer of Fermented Beverages: GMMs vs. Microbial Consortium

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Abstract

Fermented beverages are widely diverse around the world and their quality is largely based on the organoleptic characteristics developed by the metabolism of the microorganisms present during fermentation. In order to achieve controllable processes in fermented beverages along with organoleptic complexity, two divergent approaches have been followed in terms of inoculum development: (1) the inoculation of multiple microorganisms, intending to promote synergism and favor organoleptic complexity derived from the metabolic diversity, and (2) the genetic modification of a single strain with the intention that it performs multiple functions. In this chapter, we discuss these divergent approaches, their achievements and perspectives.

Keywords: microbial consortium, genetic modified microorganism, biochemical changes, fermented beverages, organoleptic characteristics

1. Introduction

The induction of fermentation on raw materials provides new products with added nutrients and organoleptic complexity vastly appreciated by consumers. The changes in the components of the raw materials are mainly caused by the main and secondary metabolism of the microorganisms present during the fermentation processes. The microorganisms need carbon and nitrogen sources to obtain energy and structural blocks to maintain cell integrity and functions and to proliferate. However, some of the carbon and nitrogen are transformed and released to the medium as by-products of the metabolism which generate the characteristics of the fermented food. Spontaneous fermentation harbors complex evolving and diverse microbiota that provides organoleptic complexity, mainly in aromas and flavors. However, it is hard to control and usually derives in inconsistent and even defective products. This is why commercial starter cultures emerged, allowing a better control of fermentation. Nevertheless, some argue that commercial inoculation

leads to a loss of unique regional style. In these cases, flavors often considered superior are achieved, at the cost of consistency and occasional production losses. The microorganism core that causes the expected characteristics of several beverages has been studied widely, indicating the participation of multiple microorganisms through different stages of the fermentation. Two divergent approaches have been proposed to improve fermentation by the controlled inoculation of multiple microorganisms each causing different expected changes in the fermentation, or by the manipulation of the genome of single strains to perform multiple tasks by themselves. Both approaches have their strengths and weaknesses, and it seems that the next step is the combination of both strategies to provide a holistic solution.

2. Metabolism in fermented beverage processes

Fermentation is the metabolic process carried out by microorganisms to obtain energy by oxidizing carbohydrates in which the final electron acceptors are organic molecules rather than O_2 [1]. The catabolism of sugars results in the production of reduced pyridine nucleotides (nicotinamide adenine dinucleotide NADH); and to regenerate it in anaerobic conditions, pyruvate acts as the electron acceptor to reoxidate NADH [2]. The different fates of pyruvate are ethanol, lactic acid, or acetate, depending on the microorganism and environmental conditions [3].

2.1 Alcoholic fermentation

Alcoholic fermentation is the transformation of the sugars, mainly glucose and fructose, into ethanol and CO_2. This process is carried out by yeast such as *Saccharomyces cerevisiae* and *S. bayanus* [4], as well as by some bacteria, including *Zymomonas mobilis*, used in Central America in the fermentation of *Agave* to produce *pulque* [5] or palm wine (Toddy) [6]. The pyruvate is decarboxylated before a final reduction by NADH, to yield ethanol. The recovery of NAD maintains the flux of glycolysis reactions [7].

In addition, other by-products of fermentation are generated, such as glycerol, acetate, succinate, higher alcohols, and esters. The production of glycerol can be considered beneficial in some cases, that is, wine production, but is undesirable in the production of distilled beverage since it represents a waste of substrate [8]. Likewise, succinate production by yeast can have an important beneficial effect on the quality of *sake*, while it produces a negative effect on wine favoring a salty and bitter taste [9]. Esters represent an important group of flavor-active compounds with beneficial fruity/floral flavors and aromas in fermented beverages [7].

It should be noted that alcoholic fermentation could occur in aerobic environments. For example, even in the presence of abundant oxygen, yeast cells greatly prefer fermentation to oxidative phosphorylation, as long as sugars are readily available for consumption, a phenomenon known as the Crabtree effect [10].

2.2 Lactic and malolactic fermentation

Lactic acid fermentation is mainly a bacterial process that plays important roles in fermented beverages, enhancing its nutritional value and organoleptic quality. A group of morphologically and physiologically diverse bacteria has been designated the term lactic acid bacteria (LAB), due to the main production of lactic acid generated from the catabolism of carbohydrates [11]. They can be divided into two physiological groups, homo- and heterofermentative, depending on the hexose metabolic pathways used. Homofermentative LAB (*Lactobacillus delbrueckii* and

Streptococcus thermophilus) ferment hexoses via glycolysis (the Embden-Meyerhof pathway), producing lactic acid as the major end product, whereas the heterofermentative LAB (*Oenococcus oeni, Lactobacillus brevis, Lactobacillus hilgardii*, and *Lactobacillus buchneri*) and facultative homofermentative bacteria *(Lactobacillus plantarum*), in contrast, ferment hexoses and pentoses via the pentose phosphate or phosphoketolase pathway to produce acid lactic, CO_2, and ethanol and/or acetic acid [12].

Malolactic fermentation, the second important stage in winemaking, normally takes place after alcoholic fermentation. The malolactic fermentation is also conducted by LAB, preferably *Oenococcus oeni*, which reduce acidity of the wine or cider by transforming malic acid (dicarboxylic acid) to lactic acid (monocarboxylic acid) resulting in a softer taste [7]. In addition, the malolactic fermentation also affects the final aroma and taste balance by modifying and producing aroma-active compounds [12].

2.3 Acetic fermentation

Acetic fermentation, also called oxidative fermentation, is a process in which alcohol is oxidized to acetic acid by the action of a group conveniently called acetic acid bacteria (AAB). These are strict aerobic bacteria found in high-sugar, alcoholic and acidic environments, characteristics found in fermented beverage processes [13]. The AAB partially oxidate carbohydrates to generate aldehydes, ketones, and organic acids in the fermentative media [14]. AAB are evidently involved in the production of vinegar and participate in fermentation of other beverages, such as palm wine, pulque, and kombucha [15]. However, the main concern with this type of microorganisms is that they are involved in the spoilage of wine, cider, and beer, where the production of acid acetic is undesired [16].

2.4 Secondary metabolism

The metabolism of microorganisms is not a straightforward pathway, and other compounds are produced in lower concentrations during the metabolization of substrates, the so-called secondary metabolites.

Higher alcohols, polyols, esters, organic acids, vicinal ketones, and aldehydes are the main secondary metabolites produced in lower concentrations, as low as ng/L, althougth human senses are able to detect them due to the low perception threshold of these compounds, providing flavor and aroma to the fermented beverages [7].

Superior alcohols, also called fusel oils, are generated as by-products of the catabolism of amino acids, specifically by transamination reaction, which yields α-keto acid that enters the Ehrlich pathway, resulting in decarboxylation forming an aldehyde, and it is then oxidized to generate an alcohol [17]. Also, the aldehyde could be released or reduced to generate an acid.

Glycerol, the most important polyol, is formed during fermentation, as one molecule of glucose at some point is divided in two molecules of three carbons, one yielding glycerol and the other pyruvate [18].

Esters are formed by the reaction of an alcohol group and an acid group. The most important are the acetate esters, in which the acid group is originated from acetic acid and ethyl esters, where the alcohol group is from ethanol. Yeast produce esters to achieve the transport from cytosol to the fermenting medium as they are able to passively diffuse the cellular membrane [19].

Vicinal diketones are formed as intermediates of the biosynthesis of branched amino acids valine, leucine, and isoleucine [20].

2.5 Microbial stress and adaptation process during fermentation

During the fermentation process, yeast and LAB must respond to several adverse conditions, mainly low pH, increasing ethanol concentration, nutrient limitations, fluctuations of oxygen concentration, and the presence of diverse compounds with antimicrobial effects [21, 22]. One of the major stress response pathways is the global stress response, including the expression of heat shock factors [23]; this is activated by several environmental conditions, as a general non-specific cell response to adverse conditions. Likewise, specific adaptation strategies are triggered under certain circumstances. Adaptation of *S. cerevisiae* environmental conditions involves the activation and repression of different sets of genes during fermentation. For example, macromolecules transport and glucose signaling are repressed at initial stages of fermentation in synthetic must, while vacuolar activity is important as far as the beginning of stationary phase [24].

Yeast viability in stationary phase is fundamental to an efficient fermentation, some reactive oxygen species (ROS) could be produced and cause oxidative damage on lipids, proteins, and nucleic acids, including mitochondrial DNA. Cells respond with the production of proteins like superoxide dismutase and rhodanases [25]. Cellular accumulation of trehalose has been associated with increased resistance to oxidative stress and survival to low temperatures [22].

Assimilable nitrogen in must have a great influence over fermentation rate in wine—low nitrogen concentration leads to a low biomass yield and slow fermentation rate [26]. During nitrogen depletion different pathways are activated such as ammonium permease, nitrogen catabolic genes, post diauxic shift elements, and autophagy; all depending of target of rapamycin signaling [27].

LAB are recognized by their high acid tolerance, and indeed, malolactic fermentation is an adaptation response to reduce wine acidity, improving its survival [28]. Other strategies to respond to high acidity are citrate fermentation, amino acid degradation to produce alkaline substances, active proton pump, accumulation of trehalose and glutathione, and degradation of phenolic acids [12].

3. Strategies to improve desirable characteristics

In the past, the main objective for the selection of microorganisms was that they achieve fermentation in a relatively short time, with high conversions from substrates to the metabolites of interest and without the generation of compounds detrimental to the quality of the fermented food [29]. Nowadays, the characteristics sought for in fermentation processes have increased to satisfy the needs of more customers and producers which aim to increase flavor and aroma rather than ethanol concentration [30]. The focus on the use of a single strain to perform such deeds is considered impossible. This is why two main strategies have been proposed and evaluated, the use of multiple microorganisms each carrying out a specific function and as a whole produce the desired change, or the use of single microorganisms genetically modified to perform several tasks by themselves.

4. Microbial consortium

During beverage fermentations, two or more microbial groups living symbiotically define a consortium [31]. In food fermentation consortia, many aspects that are summarized as follows need to be considered: (1) different strains fulfill different and complex tasks, dividing work; (2) an adequate dynamic of the interactions

between microorganisms leads to stronger adaptability and stability of the consortium; (3) the participation of different microorganisms increases complexity in microbial dynamism, metabolism, transcriptomics, and interactions, that ultimately affect organoleptic characteristics of the product. Thus, along with the evolution of the medium, these microorganisms will establish relationships that will modify their individual behavior, determining temporal dominances, proportion of the participants, and thus major metabolites, which according to the substrate, will give organoleptically complex, microbiologically stable, and healthy products that consumers desire.

4.1 Main microorganisms present in some fermented beverages and their roles

It is still unclear how much mankind has intervened in the evolution of certain groups of microorganisms in fermented foods; however, it is clear that each substrate itself exerts a different selection pressure on them. In order to determine the diversity and evolution of a microbial consortium in any type of substrates, two approaches are available nowadays. First, the traditional microbiological methods, defined as culture-dependent, which may be biased by selectivity of culture media, low populations, and the presence of viable but non-culturable cells; however, it allows to further study individual behavior of isolates. The second approach is the culture-independent or molecular methods, which nevertheless may be affected by the specificity of primers, conditions of the reaction, detection of death cells, and database availability. Culture-independent methods have allowed to obtain a more complete scene, and combining with selective flow cytometry, metabolomics, and transcriptomic studies, a further comprehensive vision of microbial biodiversity of fermented foods can be reached [32]. Some of the most important fermented beverages are presented in **Table 1**, according to the type of dominant microorganisms and the raw materials used for their preparation.

Main microorganism	Raw material (substrate)	Examples	References
Saccharomyces yeasts	Fruit	Fermented teas, wine, cider, perry, fruit-fermented beverages.	[33–35]
	Dairy	Kumis, kefir	[36]
	Grains	Beer and distillates	[37]
Non-*Saccharomyces* yeasts	Fruits	Pulque and mezcal	[5]
	Dairies	Kumis, kefir	[36]
	Grains	African fura, Mexican pozol, South American champú, Asian rice wine, among others.	[38–41]
Lactic acid bacteria	Fruits	Pulque, Taberna, tomato juice, pomegranate juice	[5, 42–44]
	Dairies	Yogurt, kefir	[45, 46]
	Grains	Sourdough, Cocoa beans, Lambic beer	[47]
Acetic acid bacteria	Fruits	Kombucha, Water kefir	[33, 48]
Molds	Grains	Sake and soy sauce	[49]

Table 1.
Classification of some of the most common fermented foods produced worldwide according to the main groups of microorganisms and the starting substrate.

4.2 Interaction between microorganisms in mixed cultures

In order to survive in an environment, a group of different type of microorganisms need to adapt and specialize through the time they spend in it. Microbial relationships are needed to establish and maintain the microbial consortium; the type of interaction that can emerge may be positive as mutualism or synergism, in which both parts benefit from being together. However, the relationships can also be negative or antagonistic, when one microorganism inhibits another, for instance by nutrients or space competition; or by producing a metabolite that harms the other; or by presenting parasitism, in which one microorganism benefits at the expense of other, damaging and even killing it [50, 51]. Any type of interaction starts by recognizing the environment, then transferring the information to others. The phenomenon is regulated by mechanisms such as quorum sensing, which consists in a stimuli-response system that regulates gene expression in response to population density [51].

In the particular environment of beverage fermentations, as exhaustively reviewed by [50], microorganisms manifest a variety of interactions. During fermentation, the environment generated maintains most of human pathogenic or food spoilage microorganisms. This role is achieved through competition and antagonism, through the fast consumption of nutrients and production of inhibitory compounds, mainly ethanol and organic acids, usually acting together with medium, short-chain fatty acids and proteinaceous toxins such as yeast's killer toxin in wine. On the other hand, throughout the evolution of the original substrate, the limiting factors change and the dominant microorganisms also change along with them. This succession of species has been reported in almost every fermented food studied. Positive interactions determine largely the succession of microbes in a particular substrate, for instance in *sake* production, where the saccharification of starch by *Aspergillus flavus* var. *oryzae* is first required in order to let *S. cerevisiae* conduct the alcoholic fermentation [52].

Besides the simply descriptive craving to know the diversity and roles that each microorganism plays, by understanding the types of interactions and how they emerge, a more controllable process can be achieved and the quality of the products can be improved. Finding the combination of microorganisms (species and strains) that will give desired characteristics is a strategy vastly explored in wine [53, 54], and also in *cachaça* [55], prickly pear wine [56] where mixed populations of *Saccharomyces*, non-*Saccharomyces* yeast, and even LAB have been explored.

One important aspect to consider when a proper combination of microorganisms is sought is to investigate their compatibility, that is, not negative type of interaction, as well as to determine if the intended promoting role actually occurs during the fermentation process. For instance, regarding compatibility, a study was conducted to observe synergism, antagonism, or no apparent interaction between selected native yeasts and LAB strains for the production of wine in the region of Queretaro, Mexico [57]. For this, yeast strains were grown in a medium resembling must, after 12 h yeast biomass was removed and the resulting broth was used to incubate the different strains of LAB and to observe their growth by means of optical density (OD) (**Figure 1**).

Positive values indicate a growth promotion from yeast to LAB observed in different extent, showing synergism superior in the combinations of native yeast strains compared with the growth promotion given by the commercial yeast (K1-V1116). It is also observable that the behaviors were strain-combination dependent, an aspect cited by other authors [58]. This test allowed to foresee and select compatible strains in order to further analyze their performance in a traditional winemaking process, where LAB strain is inoculated after the alcoholic fermentation performed by the yeast strain.

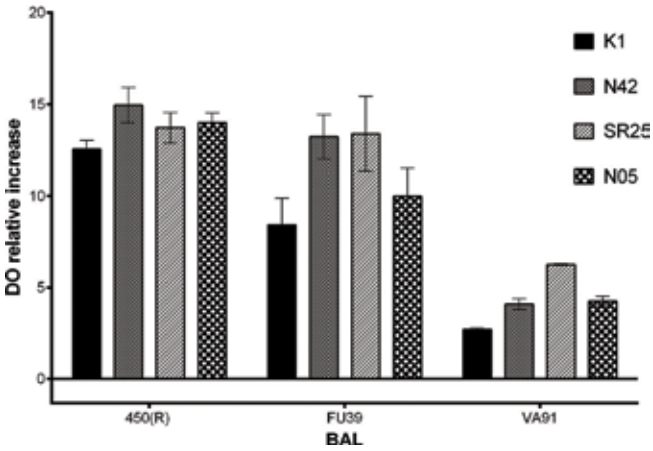

Figure 1.
Compatibility of four native LAB grown into the medium produced by five native S. cerevisiae *strains (N42, SR25, N05), measured as relative optical density increase* $\left[\text{ODi} = \frac{\text{ODi}_{\text{afteryeast}} - \text{ODi}_{\text{withoutyeast}}}{\text{DOi}_{\text{withoutyeast}}}\right]$. *Strain 450®* (O. oeni*) and K1-V1116® (K1) were used as commercial references.*

In a different context, regarding a particular metabolic interest or synergism, a study carried out in tequila fermentation is briefly presented. An important safety issue in the consumption of tequila (and in general, distillates) to take into account is the elevated concentration of ethyl carbamate generated by the reaction between urea and ethanol driven by the elevated temperatures occurring during the distillation stage. While ethanol is the desired metabolite in this process, urea is the by-product of nitrogen metabolism of *S. cerevisiae* and thus its production cannot be totally eliminated. On the other side, bacteria are capable of consuming urea as nitrogen source [59]. Taking advantage of the usual symbiosis across yeast, LAB, and AAB, an alternative approach that has been explored to reduce ethyl carbamate production is the use of mixed cultures, combining a selected *S. cerevisiae* strain and bacteria strains isolated from spontaneously fermenting agave juice (**Figure 2**).

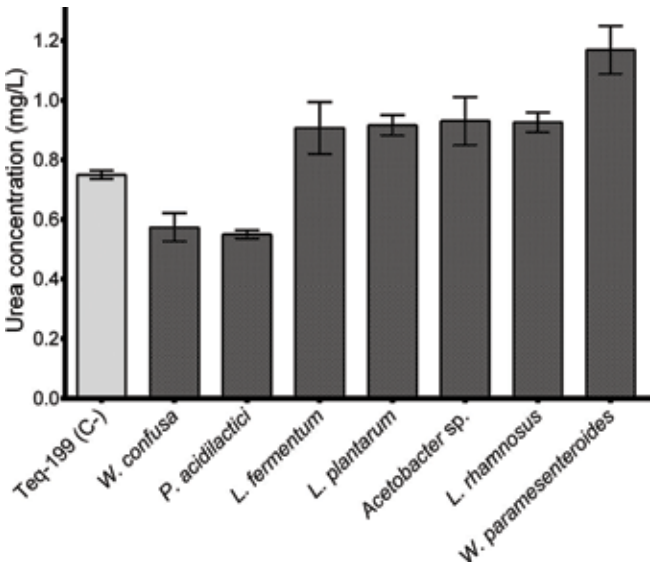

Figure 2.
Urea concentration produced by S. cerevisiae *strain Teq-199 individually (C-) or in combination with seven native bacteria species (data not published).*

Compared with fermentation individually carried out by *S. cerevisiae* strain, a clear tendency to decrease urea concentration of approximately 0.2 mg/L was observed when *Weissella confusa* and *Pediococcus acidilactici* were co-inoculated with the *S. cerevisiae* strain. Conversely, a moderate increase was obtained with the rest of the bacterial strain, especially with *Weissella paramesenteriodes*, with an increase of about 0.4 mg/L compared with the control. These changes are respectively associated with a consumption and production of the metabolite in question, depending on the species used.

These cases exemplify some of the strategies that have been followed in order to choose or validate the use of mixed cultures, seeking to achieve particular objectives and trying to ensure the success of combining certain strains.

5. Genetically modified microorganisms

Natural genetic differences are shown in strains of the same species. This variability can be replicated under laboratory conditions intended to improve characteristics of microorganisms [60]. These traits could be modified by directed or by "natural" methodologies. Even though both approaches result in genetically modified microorganisms (GMMs), the laws that dictate the feasibility on food production depend on the strategy used [61].

It is necessary to consider that the strains to be modified for food fermentation must be labeled as generally recognized as safe (GRAS) or qualified presumption of safety (QPS), not related with pathogens; so, they should be taxonomically identified, as well as being genetically stable under industrial processes [62]. Under these considerations the most investigated eukaryotic microorganism is *S. cerevisiae*, used for several centuries for food and alcoholic production; thus, their metabolic pathways and gene-related regulation are well known. Furthermore, the genome of this species has been completely sequenced, providing the basis for applications of genetic engineering [63]. Meanwhile, technological improvement investigation has been carried out mainly on LAB (**Table 2**).

5.1 Directed genetic modifications

The directed modification is carried out by genetic engineering causing a punctual manipulation in a known region in the genome that in turn will improve a characteristic of interest or the repression of a negative trait. The changes usually involve the promoter region to induce or repress gene translation, or the deletion or insertion of new genes from other microorganisms. This approach presents several drawbacks in food industry. First, it requires the global knowledge of metabolic pathways, genes involved, and their regulation [79]. Second, a single gene modification cannot produce the expected result, since some pathways are regulated by several genes, making a complex process to obtain the desirable trait [61]. And third, the use of microorganisms modified this way is prohibited in foods by law in the European Union, USA, and other countries [80].

The only permitted directed genetically engineered strain used in USA is a *S. cerevisiae* strain able to fully carry out a malo-alcoholic fermentation. This strain was generated by the integration of a malate permease gene from *Schizosaccharomyces pombe* and malic enzyme from *O. oeni* to the constitutive promoter of the 3-phosphoglycerate kinase of *S. cerevisiae* [81].

Modification technique	Species	Modified trait	Reference
Adaptive evolution	*S. cerevisiae*	Flocculation in the surface	[64]
	S. cerevisiae	Ethanol reduction and flavor increase	[65]
	S. cerevisiae	Ethanol reduction	[66]
Random mutagenesis	*L. lactis*	Domestication from plant to milk fermentation	[67]
	Yeast (species not identified)	Reduction of acetic acid	[68]
	O. oeni	Malolactic efficiency and sensory properties	[69]
Natural conjugation	*S. cerevisiae* *S. bayanus*	Fermentation at low temperature	[70]
	S. cerevisiae *S. bayanus*	Stress resistance and fermentation performance	[71]
	S. cerevisiae *S. paradoxus* *S. pastorianus*	Aroma production	[60]
	S. cerevisiae	Determine gene implicated in nitrogen requirements	[72]
	S. cerevisiae	Acid- and thermo-tolerance	[73]
Genome shuffling	*S. cerevisiae*	Improve fermentation performance, affected negatively the flocculation capacity	[74]
	S. cerevisiae	Improve fermentation performance	[75]
	S. cerevisiae	Improve fermentation performance	[76]
	Candida krusei	Improve acetic acid tolerance	[77]
	Acetic acid bacterium	Improve tolerance of ethanol	[78]

Table 2.
Examples of genetic modifications applied to microorganisms for fermented beverages improvement.

5.2 Natural genetic modifications

To obtain microorganisms with desired genetic characteristics using natural techniques, growth conditions are guided in the laboratory to improve the probability of inducing the desired genome modifications. All these natural techniques target the whole genome of the strain, generating several different genotypic changes and, thus, generating the need to further select the strains with the phenotypic variation desired. These methodologies are "allowed," or at least not prohibited by the law as they do not enter in the legal definition of GMM [30]. Among other strategies, some of the most important are described below.

5.2.1 Adaptive evolution

In this methodology, strains are grown in a medium exerting an increasing selective pressure to allow the most adapted generations to become dominant. During the replication of DNA, mutations could accumulate in the offspring without causing an evident modification. However, in a selective condition, only strains with the genetic pool needed to maintain the homeostasis of the cell under the stress pressure will be able to grow [82].

Adaptive evolution has been applied to divert ethanol to glycerol production, then reducing ethanol graduation in wine. It was achieved by increasing osmotic stress with salts in growth media. Glycerol is produced and accumulated in the interior of the yeast cell to counteract the osmotic pressure in the environment [66].

5.2.2 Random mutagenesis

The exposure of microorganisms to physical factors such as UV light, or chemical mutagens as alkylating agent, allows increasing the rate of mistakes in the replication. The offspring then are screened to select colonies with improved characteristics. The randomness of the mutations causes a big drawback, and the modification of regions other than the target of interest could impact negatively on the performance [83]. Also, as the genes occur in more than one copy in the genome, the mutation should be present in all the copies to obtain a strain with changed phenotype [84].

5.2.3 Natural conjugation

This methodology mainly has been applied to yeast, in which two strains, both having an interesting characteristic are crossed using their sexual cycle, thus also receiving the name of direct mating [60]. The resulting hybrid strain contains half genes from each parental strain, meaning that it will obtain some characteristics and lose others [85]. To discriminate the new hybrids from the parental strains, the latter must be differentiated, usually using respiratory-deficient and auxotrophic strains, which in turn only hybrids with prototrophy and respiratory proficiency would be able to grow in a selective media [60].

The most famous yeast strain generated by natural hybridization is the lager beer *S. pastorius*, having characteristics of *S. cerevisiae* and cryotolerance of *S. eubayanus*, which gave the desired fermentative proficiency at low temperatures [37]. Laboratory hybridization of *S. cerevisiae* x *S. mikatae* has also generated strains with improved and diverse volatile compounds that provide complexity to wines [86]. In addition, a hybrid of *S. cerevisiae* and *S. kudriavzevii* accumulated more glycerol, providing more cryotolerance, osmotolerance, and ethanol tolerance [87].

The major drawback of the sexual reproduction in yeast is that industrial strains poorly sporulate [61]. Rare mating is applied in these cases, switching the mating type of diploid or polyploid cells, and then being able to hybridize with the contrary mating type, to generate a new hybrid [88].

5.2.4 Cell fusion

In this methodology, the cell wall is disrupted generating spheroplasts that will spontaneously fuse to other cells, integrating their DNA into a single cell and, then, recombination occurs. The insertion of genetic material could be done even from microorganisms of other kingdoms [89].

Genome shuffling is based on protoplast fusion and nowadays several methodologies are integrated to provide complex phenotypes. It involves the induction of mutagenesis in a population of a specific strain, and then this new genetically diverse population could be screened by the evaluation of individual isolates or by applying a selective pressure to the media containing the mutants. The resulting exceptional mutants are hybridized by protoplast fusion or by mating. The resulting combinations could be further hybridized repeatedly to improve characteristics of

interest [75]. As this methodology is relatively new, their evaluation at industrial level to provide certainty of the results is still needed.

5.2.5 Horizontal gene transfer

In nature, horizontal gene transfer occurs in fungi and bacteria kingdoms, it involves the insertion of sequence elements, conjugation, transformation, and transduction from one microorganism to another [90]. These transferences could happen in non-taxonomically related microorganisms. In yeast, this mechanism is not well known; however, it has provided important features such as the identified in a *S. cerevisiae* strain by whole genome sequencing, in which a total of 34 genes were found to be transferred from non-*Saccharomyces* and *Zygosaccharomyces bailii*, providing important fructose fermentation capability [30].

Regarding bacteria, mating process involves close physical contact between a strain that donates its genetic material, mainly a plasmid, to a recipient. The vast majority of the plasmids transferred do not contain any technological use [91]. In LAB, important plasmids naturally present provide the ability to ferment lactose, gain resistance to bacteriophages, and produce bacteriocins [92]. Plasmids could also encode for antibiotic resistance and further transferring could occur to other species of importance to pathogenic bacteria [93].

6. Trends and perspectives

During the last years, there has been an increase in the demand of natural, artisanal, and organic-labeled products, leading to a rise in the request for autochthonous starters, which reflect the biodiversity of a particular area, supported by the idea of microbial "terroir."

An alternative to the use of single-strain starter cultures, which leads to very standardized products, is the use of autochthonous mixed starters (consortia), able to mimic the natural biodiversity, increasing organoleptic properties, but still maintaining controllable processes [52, 53].

On the other hand, considering the fact that mixed populations can perform functions that are difficult or even impossible for individual strains or species to do, nowadays the theoretical support to successfully obtain synthetic microbial consortium exists and presents a wider application potential than single synthetic cells. Taking into consideration the knowledge acquired on naturally occurring microbial interactions, the application of such technology seems feasible and attractive for many industries. This approach would make it possible to efficiently complete many tasks and to acquire a specific product profile compartmentalizing molecular components of each pathway, transcriptional regulators, and chemical intermediates in each different microbial individual. Nevertheless, the use of this technology would face many drawbacks until it is approved to be used in fermented foods, in spite of being the focus of several studies in other similar fields [94-96].

7. Conclusions

The genetic modification of strains and the development of mixed starter cultures aim for similar objectives, to improve the characteristics of fermented beverages maintaining control of the process and quality of the products. Both approaches possess strengths and weaknesses. While some advocate that changes in the genome open a vast opportunity to achieve all the desired characteristics in

fermented beverages, the other groups remark that only natural diversity and traditional methods could generate best products with typicity. Furthermore, the application of genetic modifications is badly perceived by consumers and legally prohibited in some cases. It seems that the next step in the improvement agenda is the combination of both approaches, the incorporation of mixtures of natural, genetically modified microorganisms and native strains to provide a holistic solution to the existing difficulties in fermentation.

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Conflict of interest

The authors state that there is not conflict of interest.

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Chapter 7

Perspectives and Uses of Non-*Saccharomyces* Yeasts in Fermented Beverages

Waldir Desiderio Estela Escalante

Abstract

Fermented beverages such as wine, cider and beer are normally fermented with *Saccharomyces* yeasts due to their well-known fermentative behavior. These yeasts have been extensively investigated and are used in commercial processes. On the other hand, non-*Saccharomyces* yeasts were always considered contaminants in winemaking and brewing. Most researchers in the past argued that these yeasts produce several compounds that may alter the sensory quality of wine and beers. However, recent studies have demonstrated that their fermentative metabolism can be regulated and addressed to the production of compounds of sensory importance. Currently, some non-*Saccharomyces* yeasts belonging to the genera *Kloeckera, Candida, Hanseniaspora* are getting importance due to their high potentiality to be used in the production of fermented beverages such as special wines and craft beers. The emergence of new consumption patterns and market niches demanding products with new sensory characteristics has catapulted the exploitation of these yeasts.

Keywords: non-*Saccharomyces* yeasts, fermented beverages, wine, craft beers

1. Introduction

Fermentation of wines, beers and ciders is traditionally carried out with *Saccharomyces cerevisiae* strains, the most common and commercially available yeast. They are well known for their fermentative behavior and technological characteristics which allow obtaining products of uniform and standard quality. *Saccharomyces cerevisiae* is the most used yeast in fermentative processes. In wine fermentation, strains with specific characteristics are needed, for instance, highly producers of ethanol to reach values of 11–13% v/v, typically found in this beverage. On the other hand, beers and ciders contain less amounts of ethanol with a balanced and distinctive sensory profile characteristic of each one. In recent years, new consuming trends and requirements for new and innovative products have emerged. This situation led to rethink about the existing fermented beverages and to meet the demands of consumers. Yeasts are largely responsible for the complexity and sensory quality of fermented beverages. Based on this, current studies are mainly focused on the search of new type of yeasts with technological application. Non-*Saccharomyces* yeasts have always been considered contaminants in the manufacture of wine and beer. Therefore, procedures for eliminating them are routinely utilized such as must pasteurization, addition of sulfite and sanitization of equipment and

processing halls. In recent years, the negative perception about non-*Saccharomyces* yeasts has been changing due to the fact that several studies have shown that during spontaneous fermentations of wine, these yeasts play an important role in the definition of the sensory quality of the final product. Based on this evidence, the fermentative behavior of some non-*Saccharomyces* yeasts is being studied in deep with the purpose of finding the most adequate conditions and the most suitable strain to be utilized in the production of fermented beverages.

2. Yeasts

Yeasts are eukaryotic microorganisms that inhabit a variety of ecological niches such as water, soil, air and the surface of plants and fruits. Commonly, they are present during the decomposition of ripen fruits and participate in the fermentation process. In this natural environment, the yeasts find nutrients and substrates necessary for their metabolism and fermentative activity [1, 2]. Yeasts are not nutritionally demanding compared to other microorganisms such as lactic acid bacteria. For supporting their growth, they need common compounds such as fermentable sugars, amino acids, vitamins, minerals and also oxygen. Morphologically the yeasts are very diverse, being the round, ellipsoidal and oval shapes mostly predominant. During the identification, the microscopic evaluation is the first resource followed by microbiological and biochemical tests; subsequently, assays of sugar fermentation and assimilation of amino acids are necessary [3]. The production and tolerance to ethanol, organic acids and SO_2 are also important tools to differentiate among species. The reproduction of yeasts is mainly by budding, which results in a new and genetically identical cell. Budding is the most common type of asexual reproduction, although cell fission is a characteristic of yeasts belonging to the genus *Schizosaccharomyces* (**Figure 1**). Cultivation conditions leading to the starvation of nutrients such as the lack of amino acids induce sporulation, which is a mechanism used by yeasts to survive under unfavorable conditions. As a consequence of the sporulation, yeast cells undergo genetic variability. In industrial

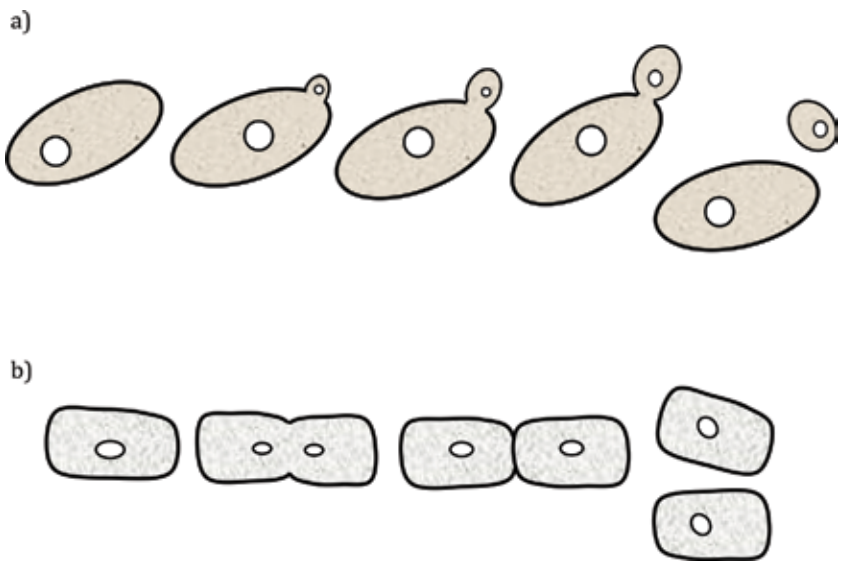

Figure 1.
Asexual reproduction of yeasts. (a) Budding, typically observed in Saccharomyces cerevisiae, Candida, Kloeckera, Brettanomyces *and (b) fission, typically observed in* Schizosaccharomyces pombe.

fermentation processes, asexual reproduction of yeasts is preferable to ensure the conservation of the genotype and to maintain their fermentative behavior over time. Regarding their metabolism, yeasts are usually characterized by fermenting a broad spectrum of sugars, among them, glucose, fructose, sucrose, maltose and maltotriose, which are found in ripen fruits and processed cereals. In addition, yeasts tolerate acidic environments with pH values around 3.5 or even less. According to technological convenience, yeasts are divided into two large groups namely *Saccharomyces* and non-*Saccharomyces*. Morphologically, *Saccharomyces* yeasts can be round or ellipsoidal in shape depending on the growth phase and cultivation conditions. *S. cerevisiae* is the most studied species and the most utilized in the fermentation of wines and beers due to its excellent fermentative capacity, rapid growth and easy adaptation. They tolerate concentrations of SO_2 that normally most non-*Saccharomyces* yeasts do not survive. However, despite these advantages, it is possible to find in the nature representatives of *S. cerevisiae* that do not necessarily present these features.

3. Non-*Saccharomyces* yeasts

Non-*Saccharomyces* yeasts are a group of microorganisms genetically diverse with specific metabolic characteristics and high potential for using in fermentation processes. In the past, many of them have been considered contaminants due to the production of compounds that alters the sensory quality of wines [4, 5]. With the purpose of eliminating them and avoiding their fermentative activity, for instance, in wine processing, disinfection of fermentation tanks and containers with sulfite is commonly performed. However, over time, the importance of non-*Saccharomyces* yeasts in spontaneous fermentation has been demonstrated since they contribute positively to the definition of the sensory quality of wines. These yeasts predominate at the initial stage of the spontaneous fermentation [6–8] until certain concentration of ethanol is reached (usually between 4 and 5% v/v), which are then inhibited due to the effect of the ethanol and the depletion of dissolved oxygen [9, 10]. At the end of the process, *Saccharomyces* yeasts, the most resistant to ethanol, predominate and complete the fermentation. It has been reported that some non-*Saccharomyces* yeasts are able to survive toward the end of the spontaneous fermentation and exert their metabolic activity, thus contributing positively to the sensory quality of wines. Based on this evidence, in recent years, many researchers have focused their studies in understanding the nature and fermentative activity of the non-*Saccharomyces* yeasts [8, 11–21]. The findings demonstrated the enormous potential of these yeasts for use in the fermentation of traditional and nontraditional beverages. Despite the fact that most non-*Saccharomyces* yeasts show some technological disadvantages compared to *Saccharomyces cerevisiae* such as lower fermentative power and production of ethanol, non-*Saccharomyces* yeasts possess characteristics that in *S. cerevisiae* are absent, for instance, production of high levels of aromatic compounds such as esters, higher alcohols and fatty acids [22, 23]. In addition, it has been reported that the fermentative activity of these yeasts is manifested in the presence of small amounts of oxygen which leads to an increase in cell biomass and the decrease in ethanol yield, a strategy that can be used to reduce the ethanol content of wines produced in coculture with *S cerevisiae* [24–26]. With the aim of exploiting the positive characteristics of non-*Saccharomyces* yeasts and reducing their negative impact, fermentations with mixed and sequential cultures with *S. cerevisiae* can be performed to produce fermented beverages with different sensory profiles [27–29]. The most important fact is related to the potential for producing a broad variety of compounds of sensory importance necessary to improve the organoleptic quality of wines and beers. The findings reported so far in literature have led to rethink the role of these yeasts in

fermentative processes and to evaluate their use in the development of new products. Among the most studied non-*Saccharomyces* yeasts that reached special importance for researchers include *Candida*, *Kloeckera*, *Hanseniaspora, Brettanomyces, Pichia*, *Lanchacea* and *Kluyveromyces*, among others.

3.1 Fermentative metabolism of sugars

Either non-*Saccharomyces* or *Saccharomyces* yeasts share common pathways for the central metabolism of carbon; thus, both groups metabolize glucose through glycolysis. However, the mechanisms involved in the regulation of respire-fermentative metabolism can differ significantly among them [30]. The glycolysis operates indistinctly under aerobic and anaerobic conditions, and through it, the glucose is metabolized to pyruvate by means of a series of biochemical reactions (**Figure 2**). Under anaerobic or oxygen-limited conditions, pyruvate is converted to acetaldehyde and then to ethanol, and as a result, two net moles of ATP are generated. Under fully aerobic conditions and in the absence of any repression effect, the

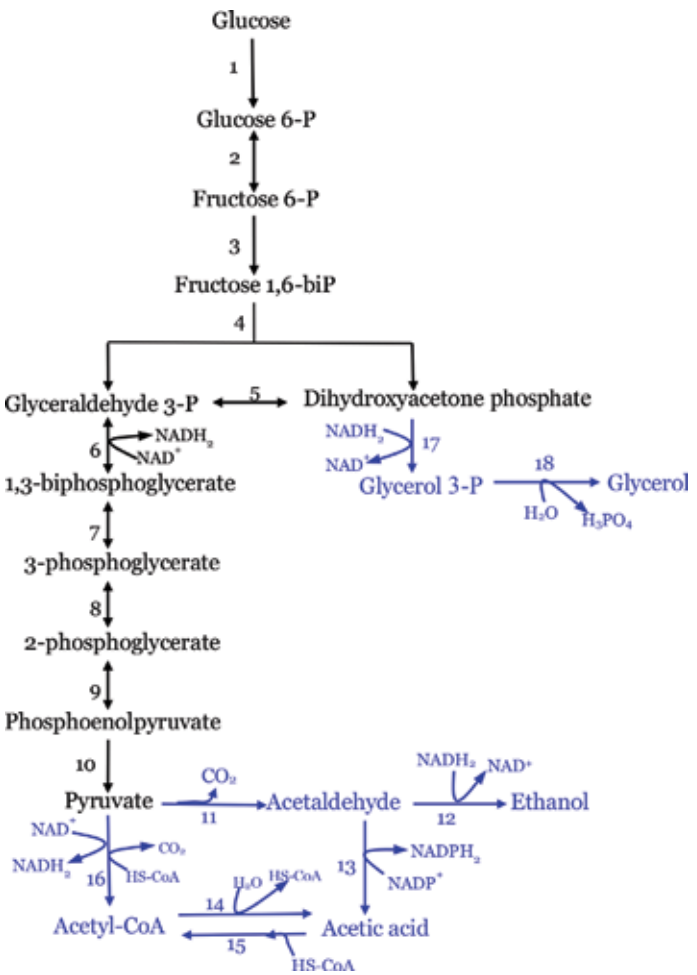

Figure 2.
Fermentative metabolism of glucose by yeasts: Glycolysis (black lines) and ethanol and glycerol production (blue lines). Enzymes: 1, hexokinase; 2, phosphoglucose isomerase; 3, phosphofructokinase; 4, fructose 1,6-bisphosphate aldolase; 5, triosephosphate isomerase; 6, glyceraldehyde 3-phosphate dehydrogenase; 7, phosphoglycerate kinase; 8, phosphoglycerate mutase; 9, enolase; 10, pyruvate kinase; 11, pyruvate decarboxylase; 12, alcohol dehydrogenase; 13, aldehyde dehydrogenase; 14, acetyl-CoA hydrolase; 15, acetyl-CoA synthetase; 16, pyruvate dehydrogenase; 17, glycerol 3-P dehydrogenase; 18, glycerol 3-phosphatase.

generation of energy is greater since glucose undergoes a complete oxidation, and as a result 36 net moles of ATP per mole of glucose are generated. The low-energy yield obtained by yeasts under anaerobic conditions forces the cell to increase the flow of glucose consumption in order to obtain a higher amount of energy in the form of ATP. As consequence, the ethanol accumulates in the fermentation medium and exerts its inhibitory effect, thus stopping the fermentative activity of the yeasts [31]. The low amount of energy generated under anaerobic conditions is used by the yeast cells in requirements for maintenance and growth. Glucose is easily transported and metabolized inside the cell; however, disaccharides such as sucrose, maltose or lactose must be first hydrolyzed to their simple forms (hexoses) which are then catabolized in the glycolysis pathway. Sucrose is hydrolyzed to fructose and glucose, maltose to two glucose units and lactose to glucose and galactose. The disaccharides are preferably hydrolyzed in the periplasmic space before entering the cytosol. Under anaerobic conditions besides ethanol, glycerol is also produced, thus contributing to restore the redox balance inside the cell. The production of glycerol increases in fermentations with musts of high specific gravity as a response to the osmotic stress [32]. It has been found that yeasts unable of metabolizing dihydroxyacetone (**Figure 2**) are not capable of producing glycerol, and as a consequence dihydroxyacetone accumulates and inhibits the fermentation. Moreover, glucose apart of being metabolized via glycolysis, it is also broken by complementary pathways that are not necessarily related to the generation of energy. The hexose monophosphate pathway (HMP) also known as the pentose phosphate cycle usually accompanies the glycolytic pathway [33]. In addition, yeasts during fermentation produce small amounts of acetic acid either from acetaldehyde or acetyl-CoA (**Figure 2**). Acetic acid is the main organic acid produced by yeasts during the fermentation of glucose, and it is responsible for the acidification and the decrease of pH of the medium. Ethanol is the most important fermentation by-product, and from the technological point of view, the production capacity of yeasts is an important parameter that determines their usability in fermentative processes. Gay Lussac defined a stoichiometric theoretical relationship to explain the production of ethanol by *Saccharomyces cerevisiae* yeasts which is:

$$\underset{\text{glucose}}{1\ C_6H_{12}O_6} \rightarrow \underset{\text{ethanol}}{2\ C_2H_5OH} + \underset{\text{carbon dioxide}}{2\ CO_2} \quad (1)$$

According to this relationship, from 180.0 grams of glucose, 92.0 grams of ethanol and 88.0 grams of carbon dioxide are produced, which results in a theoretical yield of 0.511 g ethanol/g glucose. However, in practice, besides ethanol and CO_2, the production of biomass, glycerol and other minority compounds also happens, that is:

$$C_6H_{12}O_6 + \text{nitrogen} \rightarrow C_2H_5OH + CO_2 + \text{glycerol} + \text{biomass} + \text{minority compounds} \quad (2)$$

At industrial scale, a yield of 0.45 g ethanol/g glucose is acceptable [34]. In the case of fermentations with non-*Saccharomyces* yeasts, lower yields are commonly observed. Regarding to glycerol, in the case of *S. cerevisiae,* its production represents approximately 3% of the utilized sugar. Minor compounds are represented by higher alcohols, esters, aldehydes and organic acids, among others.

3.1.1 Importance of oxygen

Oxygen is an important element during the complete oxidation of glucose since it serves as final acceptor of electrons under aerobic conditions. It is also essential for other metabolic processes such as the synthesis of structural components of the

cytoplasmic membrane of yeasts. During alcoholic fermentation, as ethanol accumulates, it exerts a detrimental effect on the integrity and stability of the cytoplasmic membrane [31]. Under this condition, the supply of small amounts of oxygen to the medium through aeration promotes the synthesis of unsaturated fatty acids and sterols (mainly ergosterol) which are important components of the yeast cell membrane. Thus, the produced compounds can be used to replace the damaged fraction caused by the effect of ethanol that acts as a solvent [35, 36]. The replacement of unsaturated fatty acids and sterols is important to maintain the cell viability and allow the yeasts to complete successfully the fermentation. From the technological point of view, the supply of small amounts of oxygen is recommended in fermentations with musts of high specific gravity in order to avoid some drawbacks such as sluggish fermentation. It is also necessary for promoting the fermentative metabolism of non-*Saccharomyces* yeasts which are unable to ferment under fully anaerobic conditions [37]. The optimization of the aeration rate is very important to ensure the predominance of the fermentative metabolism and to reach the highest ethanol yield. In Crabtree-negative yeasts, as the concentration of oxygen in the medium increases above a certain value, the metabolism may become predominantly oxidative; thus, the ethanol yield decreases and the production of biomass increases. The highest ethanol yield is possible to achieve, adjusting properly the aeration rate of the fermentation medium. Aeration also affects the production of glycerol by yeasts; thus, as the concentration of oxygen increases, the production of glycerol decreases. From the technological point of view, aeration of the fermentation medium is an interesting tool to control the metabolic activity of non-*Saccharomyces* yeasts during fermentation, for instance, wines and beers [38, 39]. In addition, aeration can be also used in winemaking to improve the quality of wines since it provokes the transformation of phenols, which reduces the astringency.

3.2 Production of higher alcohols

During alcoholic fermentation, either non-*Saccharomyces* or *Saccharomyces* yeasts produce diverse volatile compounds of sensory importance such as higher alcohols, aldehydes, fatty acids and esters in different concentrations depending on the species of yeasts and the fermentation conditions. The harmonic balance of the compounds determines the sensory quality of the fermented beverage. Higher alcohols are a group of compounds that mostly confer unpleasant organoleptic character when present at high concentrations [40, 41]. In adequate concentrations, they contribute positively in defining the organoleptic quality of alcoholic beverage such as wines, beers and ciders. They are produced in the cytosol and then exported outside the yeast cell where it accumulates. Higher alcohols result from the decarboxylation of ketoacids that leads to the formation of the respective aldehydes, which are then reduced to form the corresponding higher alcohols (**Figure 3**). Ketoacids can be originated either from the metabolism of glucose or the catabolism of amino acids [42, 43], which are taken by the yeast cell from the fermentation medium. The synthesis of higher alcohols involves the participation of at least three enzymes: a transaminase, a carboxylase and an alcohol dehydrogenase. Factors that increase the metabolism of sugar and amino acids promote the synthesis of higher alcohols. The factors include temperature of fermentation, amino acid concentration and composition of the fermentation medium.

3.3 Production of esters

Esters are a group of compounds that mostly impart positive sensory characteristics to fermented beverages such as wines, beer and ciders. They are formed by the

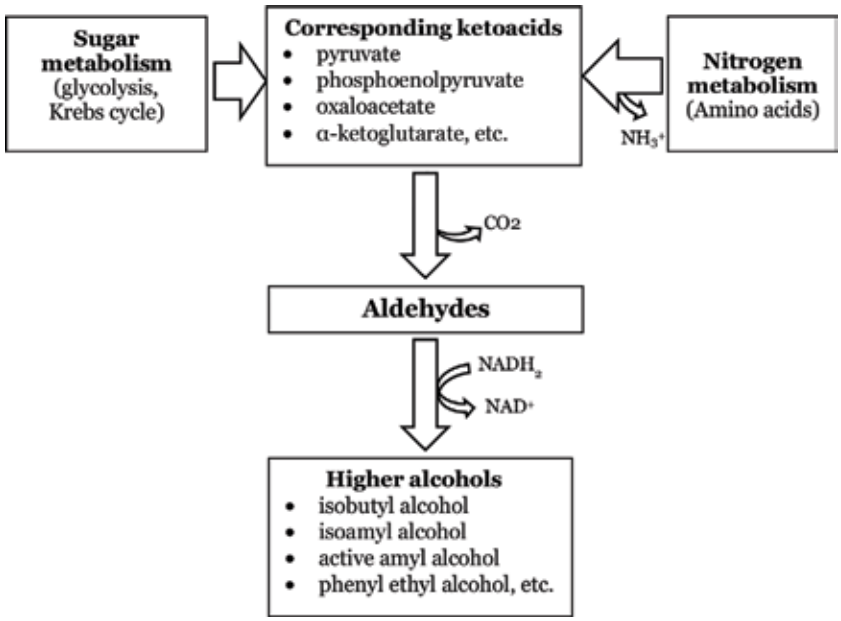

Figure 3.
Production of higher alcohols by yeast. Ehrlich's pathway and glucose catabolism.

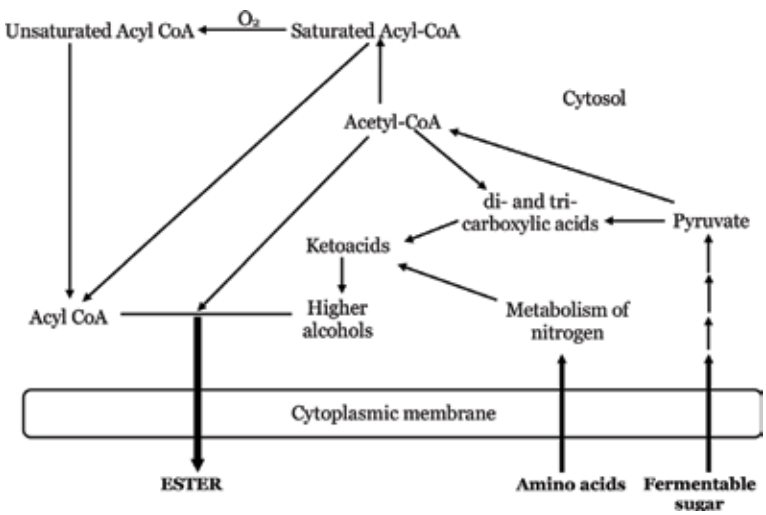

Figure 4.
Mechanisms for the production of esters by yeasts.

action of specific enzymes that catalyze the reaction between an alcohol and a volatile fatty acid (**Figure 4**). The synthesis of esters by yeast initially involves the activation of fatty acids to acyl coenzyme A mediated by energy and the subsequent condensation of the active compound with an alcohol present in the medium to form the corresponding ester [44]. From the sensory point of view, acetate esters are the most important compounds present in fermented beverages, which include ethyl acetate, butyl acetate, propyl acetate, phenyl ethyl acetate and amyl acetate, among others. The esters produced by *S. cerevisiae* involve the activity of at least three acetyltransferases (AAT, EC 2.3.1.84): an alcohol acetyltransferase, an ethanol acetyltransferase and an isoamyl alcohol acetyltransferase [45, 46]. Other enzymes such as ester synthase were also reported to participate in the synthesis of esters.

However, the relevance attributed to the activity of this enzyme is quite limited. Ethyl acetate is the most abundant ester present in wines and largely responsible for the sensory character. Studies carried out with non-*Saccharomyces* yeasts related to the ability of producing esters allowed to select species of *Hanseniaspora* and *Pichia* able to promote esterification of various alcohols such as ethanol, isoamyl alcohol and 2-phenyl ethanol to produce the corresponding esters [47].

4. Most important non-*Saccharomyces* yeasts

4.1 *Candida* yeasts

In the last years, the fermentative behavior of some *Candida* yeasts has been studied with respect to the production of wines and beers. The most studied species include *Candida stellata*, *C. zemplinina* and *C. pulcherrima*, among others [16, 20, 21, 48–50]. Representatives of *Candida* yeasts have been isolated from the early stages of spontaneous fermentation of different types of wines [8, 19, 51, 52]. The isolated species were characterized by being round in shape and smaller than *S. cerevisiae*. These yeasts are able to sediment toward the end of fermentation in a similar manner as *S. cerevisiae* [20]. Currently, the most important characteristics reported include the production of considerable amounts of ethanol and glycerol and a balanced production of volatile compounds of sensory importance, for instance, esters, fatty acids, aldehydes and higher alcohols. The production of ethanol is an important feature to define the use of yeasts in the production of fermented beverages with high ethanol contents such as wines. It has been reported that *C. zemplinina* strains are capable of producing ethanol up to 11.0% v/v [53], amount normally reached during the fermentation of sweet and semidry wines with *S. cerevisiae*. In addition, it has been demonstrated that *Candida* yeasts are capable of producing high amounts (up to 25.0 g/L) of glycerol [53–56], compound that contributes positively to the sensory quality of wines, beers and other beverages. The fermentative behavior of these yeasts was also evaluated as mixed cultures with *S. cerevisiae* [57]. The results were promising and interesting for being scaled-up to pilot fermentations. For instance, fermentation experiments of mixed cultures of *C. stellata* with *S. cerevisiae* produced higher levels of esters and fatty acids than monocultures of *S. cerevisiae* [19, 57]. Fermentations with mixed and even sequential cultures of yeasts are an interesting field of research to evaluate the potential use of non-*Saccharomyces* yeasts to produce sensory differentiated beverages. In addition, individual fermentations with *C. stellata* and *C. zemplinina* strains using immobilized systems have been also performed [53, 58]. The results showed the improvement of some technological properties such as the fermentation rate, ethanol production and the reusability of the strains in successive fermentations. Currently, studies to evaluate the usability of *C. zemplinina* strains in beer fermentation have been carried out using malt wort of 14 and 20°P, typically used in beer fermentation processes [21, 22]. The yeast strains showed a suitable fermentative behavior for the production of lager and ale beers. One interesting feature is that *Candida zemplinina* is unable to ferment maltose, the main fermentable sugar of the malt wort. This characteristic is of special importance since it would enable the production of beers with low ethanol content and particular sensory profiles.

4.2 *Kloeckera* yeasts

Yeasts species belonging to this genus have recently become of interest for the production of fermented beverages. Species such as *Kloeckera apiculata, K. javanica*

and *K. corticis* were isolated from a variety of niches including the spontaneous fermentation of grape must and ciders [6, 8, 49, 59]. Most representatives present a lemon shape (apiculate yeasts) and asexual reproduction with bipolar budding. It was reported that these yeasts participate positively in the early stage of the spontaneous fermentation of wine [59, 60], strains of *Kloeckera apiculata* being the most dominant [19, 49, 51, 52]. During spontaneous fermentation, as the ethanol concentration increases, the fermentative activity of these yeasts slows down and stops toward the end of fermentation by the effect of the ethanol [61]. These yeasts are characterized by producing amounts of ethanol around 4–5% v/v, values typically found in commercial beers. It was reported that the control of aeration during fermentation has effect on the production of ethanol and compounds of sensory importance such as esters, higher alcohols and organic acids [14]. Based on the information available in literature, these yeasts are promissory for being used in brewing; however, before defining a strategy of exploitation, it is necessary to carry out more in-depth studies on the effect of temperature, wort composition and inoculation rate in the fermentative activity of these yeasts. In addition, it is also necessary to carry out studies on the behavior of these yeasts in fermentations with mixed and sequential cultures with *Saccharomyces cerevisiae* and the production of compounds of sensory importance. Studies carried out with pure cultures of *Kloeckera corticis* showed that these yeasts are capable of producing acetic acid, acetaldehyde, ethyl acetate and acetoin at high concentrations [62]. In addition, it has been reported that strains of *Kloeckera apiculata* are capable of producing higher concentrations of ethyl and isoamyl acetate than other non-*Saccharomyces* yeasts [14, 63]. From the technological point of view, techniques of cell immobilization can be an additional strategy to improve the fermentative behavior and the production of compounds of sensory importance. The ability of these yeasts to produce a variety of aromatic compounds with positive impact on the sensory quality makes them attractive and potentially exploitable in fermentation processes.

4.3 *Hanseniaspora* yeasts

Few studies have been conducted regarding the potential use of yeasts belonging to the genus *Hanseniaspora* (apiculate yeasts) in the production of fermented beverages. The studied yeasts were isolated from the spontaneous fermentation of grape musts [6, 8, 59] and include species of *Hanseniaspora uvarum*, *H. osmophila* and *H. guilliermondii*, among others. It has been shown that these yeasts play an important role during the early stage of spontaneous fermentation of wine and strains of *Hanseniaspora uvarum* (also called *Kloeckera apiculata*) are dominant [19, 51, 52]. They are characterized by tolerating and producing low amounts of ethanol that do not exceed the values of 5.0% v/v [61]. This limitation explains why these yeasts do not participate actively toward the end of spontaneous fermentation of wines where the ethanol content reaches values even higher than 10%v/v. However, the fermentative capability of these yeasts is enough to produce beers of standard ethanol content similarly to those found in the market (4.5–5%v/v). In addition, they are able to ferment a wide range of sugars including maltose, which is an important feature needed for the production of beers. Regarding the production of compounds of sensory importance, studies have reported that strains of *Hanseniaspora osmophila* are characterized by producing high concentrations of acetic acid, acetaldehyde and ethyl acetate [62]. Additionally, it was also found that strains of *Hanseniaspora uvarum* are able to produce a variety of esters that confer fruitiness to fermented beverages [11, 62, 64]. However, other studies reported that mixed cultures of *H. uvarum* with *S. cerevisiae* produce higher amounts of higher alcohols than monocultures with *S. cerevisiae*

[4, 19]. Regarding fermentation parameters, the control of aeration and temperature exerts an important effect on the dynamics and activity of *Hanseniaspora* yeasts. Both parameters are important to control the production of compounds of sensory importance, which influence the quality of fermented beverages [11, 65]. However, in view of the scarce information on the fermentative behavior of *Hanseniaspora* yeasts, particularly referring to the production of fermented beverages, additional studies are needed to perform in order to find the adequate conditions for their usage, for instance, in the production of beers with new sensory profiles.

4.4 *Brettanomyces* yeasts

Yeasts of this genus do not have a good reputation in fermentation processes such as in winemaking. For instance, representatives of *Brettanomyces bruxellensis* are considered detrimental due to the production of compounds such as 4-ethylguaiacol, 4-ethylphenol and 4-ethylcatechol which impart unpleasant sensory character to wines known as "Bretty" [5, 66]. These compounds result from the activity of a decarboxylase that acts on hydroxycinnamic acids followed by a reduction reaction [67]. The hydroxycinnamic acids are phenolic compounds naturally present in the skin and seeds of grapes. The common representatives of this genus were isolated from the spontaneous fermentation of wine, beer, cider and even kombucha [68–70]. It was also isolated from equipment and utensils utilized in fermentation processes, which are difficult to sanitize. The commonly isolated species include *Brettanomyces bruxellensis*, *B. lambicus*, *B. intermedius* and *B. anomalus*, among others [68, 69]. Particularly, strains of *B. bruxellensis* are able to ferment only in the presence of oxygen (positive Crabtree effect), a broad spectrum of sugars and even maltooligosaccharides which are not fermentable by *S. cerevisiae* [71]. Under anaerobic conditions, these yeasts are unable to ferment and produce ethanol; thus, at low concentration of sugar in the medium, the fermentation of glucose to ethanol is blocked. On the contrary, the fermentation is stimulated in the presence of oxygen, an effect known as Custer or negative Pasteur [72]. Apart from producing ethanol in the presence of oxygen, *Brettanomyces bruxellensis* also produces high concentrations of acetic acid, which acidifies and lowers the pH of the medium. However, yeasts of this genus are not entirely undesirable; some representatives participate, for instance, during the fermentation of certain beers known as "Lambic" and "Gueuze" consumed commonly in Belgium and "Coolship Ales" in North America. The fermentation of "Lambic" beer is a spontaneous process which goes through a complex succession of microorganisms where *Brettanomyces bruxellensis* participates during the final stage acidifying the product [73]. The participation of these yeasts gives the beer its characteristic acidity and dryness and additionally is responsible for the production of compounds such as ethyl phenol, ethyl acetate, ethyl caprylate, ethyl decanoate and ethyl lactate, which synergistically confer their typical aroma character [18, 74]. It has been shown that esters soften the sour taste and add fruity notes to this kind of beers [75]. Based on these findings, it was demonstrated that these yeasts and particularly *B. bruxellensis* contribute positively to defining the floral and fruity character of "Lambic" beers [18]. Beyond the contribution of *Brettanomyces* yeasts in spontaneous fermentation processes, in recent years, their use in controlled fermentations has been investigated, both in pure and in coculture with *S. cerevisiae* [15, 17]. Interesting findings were reported, indicating that the control of aeration during fermentation is a critical point to guide the fermentative metabolism toward the production of important volatile compounds that may contribute to the organoleptic character of fermented beverages.

5. Production of special wines

It is of common agreement that non-*Saccharomyces* yeasts contribute beneficially to the sensory quality of spontaneously fermented wines, an evidence that served as a starting point to pay attention to particular yeast species that could be exploited in fermentations of commercial and noncommercial fermented beverages. Non-*Saccharomyces* species are characterized by producing a greater diversity of compounds of sensory importance than *S. cerevisiae* yeasts. Although these yeasts show a low fermentation power, some species possess important fermentative features, for instance, representatives of *Kloeckera* and *Hanseniaspora* yeasts produce a variety of compounds of sensory impact, particularly esters at concentrations even higher than *S. cerevisiae*. On the other hand, *Candida zemplinina*, a fructofilic yeast, has been shown to produce glycerol in higher concentrations than *S. cerevisiae*. It is also capable of producing ethanol in concentrations high enough to produce different types of wines. In view of the complementary characteristics of both groups of yeasts (*Saccharomyces* and non-*Saccharomyces*), the use of non-*Saccharomyces* yeasts can be proposed in fermentations with mixed or sequential cultures with *S. cerevisiae* as an important strategy to improve sensory complexity and mouthfeel of wines [19, 73]. The fermentative versatility of non-*Saccharomyces* yeasts would enable the production of special wines with different and innovative sensory characteristics. In addition, among the techniques that can be implemented for enabling their practical exploitation include the selection of new strains, the development of fermentation strategies (mixed or sequential cultures with two or more yeast strains), the ratio of both strains in the inoculum (non-*Saccharomyces*/*Saccharomyces cerevisiae*) and the inoculation rate at the beginning of fermentation [57, 76]. Finally, some technological characteristics of non-*Saccharomyces* yeasts can be also modified by using cultivation techniques in bioreactors with the aim of improving, for instance, the fermentation rate. The possibility of commercializing as starter cultures is an attractive opportunity for the production of different types of wines with special sensory qualities.

6. Production of craft beers

In the last 10 years, the market of craft beers has increased in the USA, Latin America and some countries of Europe [77, 78]. This phenomenon is related to the expectation of consumers for discovering in these beers sensory characteristics different from those routinely found in commercial beers [74]. Current consumers are curious and interested in sensing new flavors and aromas that can satisfy their preferences. As consequence, new market segments have emerged in response to the broad possibility of offering new types of beers produced using different methods and techniques of fermentation. The production of craft beer is generally carried out in small-scale breweries and involves the use of non-technified processing methods. Craft beers are not usually filtered; due to this, their shelf life is relatively short, and therefore, their consumption must be within few days after bottling. There are a variety of innovative alternatives to produce different types of craft beers which include the use of new types of adjuncts either amylaceous (cereal grains) or non-amylaceous (fruit pulps or juices) and selected strains of non-*Saccharomyces* yeasts which have an enormous exploitation potential. Although most non-*Saccharomyces* yeasts produce low concentrations of ethanol, the fermentative capacity of some representatives of *Kloeckera* and *Hanseniaspora* yeasts is adequate to produce beers with an ethanol content typically found in the market

(4.5–5%v/v). Among non-*Saccharomyces* yeasts considered important in beer fermentation, *Brettanomyces lambicus* is the most representative which is involved in the production of "Lambic" and "Gueuze" beers. Currently, some studies with *Candida zemplinina* strains were performed in fermentations with pure malt wort and with different adjuncts (grape or apple juice) at different temperatures and specific gravities. The findings were promissory and showed the capability of these yeasts to ferment at low temperatures (14°C) and in medium with high specific gravity (16°P), which demonstrates the possibility for being exploited in the production of craft beers. In addition, it was also proposed that these yeasts can be used for the production of beers with low ethanol content since they are not able to ferment maltose, the main and most abundant sugar present in the wort [20, 21]. Additionally, other non-*Saccharomyces* yeasts such as *Dekkera anomala, Naumovozyma dairenensis* and *Debaryomyces* spp. have been also reported with a high potential for being used in the fermentation of beers. In view of the different fermentative behavior of non-*Saccharomyces* yeasts and the variety of compounds of sensory importance that they can produce during fermentation, their use in controlled fermentations has aroused the interest of brewers for producing beers with distinctive sensory features [23, 79].

7. Conclusion

Non-*Saccharomyces* yeasts show a great potential to be used in the production of fermented beverages mainly wines and beers. These yeasts show a variety of fermentative patterns, and depending on the fermentation conditions, they produce a wide range of volatile compounds of sensory importance. For their practical application in a particular fermentative process, it is necessary knowing the parameters that directly influence on the fermentative activity and the production of desirable volatile compounds. Among the non-*Saccharomyces* yeasts that have attracted interest of researchers due to their fermentative qualities include strains of *Candida stellata, C. zemplinina, Kloeckera apiculata* and *Hanseniaspora uvarum*. Particularly, strains of *Candida stellata* and *C. zemplinina* have become very attractive for using in fermentations of different types of wines and beers. These yeasts are capable of producing significant concentrations of glycerol, an important compound that imparts a positive impact on the sensory quality of wines and beers. *Candida* yeasts, especially *C. zemplinina*, also produce high concentrations of ethanol, high enough to drive fermentation processes of wines. On the other hand, species of *Kloeckera* and *Hanseniaspora* yeasts are characterized by producing considerable amounts of acetate esters, valuable compounds that contribute positively to the sensory character of beers. Based on this, if a fermentation process that involves the use of non-*Saccharomyces* yeasts is going to be implemented, it is necessary to select the best representatives and then define the appropriate fermentation conditions for the production of fermented beverages with the desired sensory qualities.

Conflict of interest

The author certifies that he has *no* affiliation with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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Chapter 8

Torulaspora delbrueckii: Towards Innovating in the Legendary Baking and Brewing Industries

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Abstract

Baking and brewing are among the oldest bioprocesses refined by human societies. Both fermentative processes have successfully used domesticated strains of *Saccharomyces cerevisiae* in their process as the biocatalyst throughout their evolution. However, the dominance of *S. cerevisiae* has limited the capability for diversification of many organoleptic properties of the final product, such as aroma and flavours. The use of non-*Saccharomyces* yeasts can be an enormous source of opportunities for innovation in both fermentative processes. *Torulaspora delbrueckii* is a ubiquitous yeast species, and numerous strains have been isolated from many different bioprocesses. The strains of *T. delbrueckii*, once considered microbial contamination, have recently shown several advantages over *S. cerevisiae* strains, including higher ethanol tolerance; better capabilities to consume wort sugars; higher resistance to hop/pH/osmotic stress; and freeze-thaw resistance, among others. This chapter aims to present a comprehensive review of frontier research on *T. delbrueckii* regarding its potential and prospects for the baking and brewing industries.

Keywords: alcoholic beverages, beer production, baking industry, brewing industry, *Torulaspora delbrueckii*

1. Introduction

Bread and beer are among the oldest foods in the human history. The consumption of both of these fermented products has been rooted as a basic human food, and at the present time, these are still among the most consumed foods around the world. The ubiquity of their production allowed a diversification and development of refined, artisan techniques, which currently comprises innumerable recipes [1, 2]. All the recipes include essentially the same basic ingredients such as cereals, yeast, and water. However, the organoleptic properties (aroma, flavours, etc.) of the final product differ greatly between recipes.

Since early times, both cereals and water were identified as fundamental ingredients for the preparation of beer or bread. Despite the fact that these ingredients have been recognised as essentials for centuries, the experimental approaches developed by Pasteur during the mid-nineteenth century revealed the existence of a third element much more essential to the fermentation process: yeast. The fermentation performed by yeast is undoubtedly the oldest and the largest biotechnology

application. There are many types of yeast strains used for fermented foods commonly known as commercial strains: baker's yeast in bread production and brewer's yeast in beer fermentation. After centuries of selection, due to the refinement of the fermentation processes, today it is easy to find a wide variety of dedicated yeast strains that are suitable for different types or styles for either beer or bread.

The yeast strains responsible of these fermented foods are able to ferment sugars present in the flour or in the wort (starch, glucose, fructose, sucrose, and maltose, among others), which is concomitant with the production of other molecules such as CO_2, the main causes of dough leavening and the natural carbonation of beer. The type of yeast strain used in the fermentation process also greatly influences the properties displayed in the final product such as the texture of the dough and the flavour, and ideally, certain yeasts can also add some nutritional values to the final product [3].

The capabilities of the yeast strains to grow fast (fast propagation) and to produce a valuable product are still currently exploited by the industrial field. Depending on the final product, the yeast strains also possess other advantages, such as high tolerance to stresses caused by high sugar concentration or the drying/freezing of dough present in baker's yeast [2]. On the other hand, brewer's yeast which is similar to wine's yeast offers particular characteristics to the fermentation process such as an optimal flocculation, high ethanol tolerance, and rapid growth at high osmolarity [4].

Since the beginning, the food industry has mainly used *Saccharomyces* or closely related strains, which could be a result of the ubiquity of this genus. As consequence, scientific research efforts have been focused mainly on *Saccharomyces cerevisiae*, a yeast that nowadays is still the most commonly used microorganism in baking, brewing, and other fermented foods [5, 6]. Furthermore, *S. cerevisiae* has also been historically used as a model organism for the research on fundamental aspects of eukaryotic cell biology. The popularity of *S. cerevisiae* in the food industry has led to the standardisation of the organoleptic properties of the final product, which limits the capability to improve or to create new properties for the final product.

The use of non-*Saccharomyces cerevisiae* yeasts, also known as "nonconventional" or "non-commercial" yeast, offers new alternatives for the development of products with improved properties, such as biomass yield, distinct flavour complexity, aroma profile, and other advantages. Among nonconventional yeasts, *Torulaspora delbrueckii* (formerly *Saccharomyces rosei* or *S. rosei*) has been considered of oenological interest for decades.

At the present time, *T. delbrueckii* is starting to be recognised as a model organism of study because most of its biological features, like its sugar metabolism, differ greatly from the more prevalent *S. cerevisiae* [7]. These differences provide many advantages of biotechnological importance in several fermentative processes; for example, it is quite interesting how *T. delbrueckii* can adapt its physiological state in order to endure the harsh conditions present during the frozen dough preservation [8]. On the other hand, the use of *T. delbrueckii* in the brewing industry has had a positive effect on the aroma and taste of the final alcoholic beverage [9–11]. The industrial brewing process has appreciated several features of this yeast strain that also complement its suitability for the fermented food preparation processes, such as its higher ability to retain cell viability under stress conditions, high ethanol tolerance, an increased osmotic resistance, and the advantageous capability to yield a final product with lower levels of unpleasant acetaldehyde, acetoin, acetate, and ethyl acetate [12]. Fermentation with *T. delbrueckii* also provides an excellent example of improvement in the industrial process with the consequent creation of new value-added products. In the present chapter, we review the current and potential applications of *T. delbrueckii* strains in the bread and brewing industries discussing their physiological perspective.

2. The genetics of *Torulaspora delbrueckii*: an overview

Torulaspora delbrueckii has unique abilities when compared to other yeasts; the use of this microorganism has many advantages yet to be potentially exploited in the biotech industry. The progress in understanding its physicochemical and physiological mechanisms of action has been hampered by the lack of genetic and molecular tools specific for this yeast. The development of tools for metabolic engineering of a specific microorganism is essential for the improvement of industrial processes. Once the microorganism of interest has been isolated and identified, one of the first steps for the development of the genetic tools is to determine its exact genome sequence. With the advent of modern, state-of-the-art equipment and facilities dedicated to unravel the DNA sequence from diverse biological samples; nowadays, the sequencing of small genomes (<50 MB) has become even cheaper and more reliable than just a decade ago.

A pioneering work presented in 2002 described the isolation of genes from *T. delbrueckii* and their subsequent heterotrophic expression using *Saccharomyces cerevisiae* as a host [13, 14]. From this work, the first yeast genomic library of *T. delbrueckii* PYCC532 was derived. In 2011, a draft genome sequence of *T. delbrueckii* CBS 1146 was published as part of a molecular evolution study [15]. The CBS 1146 strain has been preserved since 1970 under laboratory conditions and thus raises the question regarding the potential differences when compared to a fresh strain isolated from an active alcoholic fermentation. A complementary study that addressed this question was published recently in 2015 [16], where a set of differential genes were identified. The most recent sequencing projects have had much better coverage than preliminary studies. Most recently, other *T. delbrueckii* genomes have been published (**Table 1**). For example, the first complete genome map of *T. delbrueckii* COFT1 has

Strain	Source	GenBank accession	Genome assembly level	Reference
CBS 1146 (NRRL Y-866)	Unknown. Isolated by the Wallenstein Lab., No. 129, 119,077. Deposited by the NRRL	GCA_000243375.1	Chromosome	[15]
COFT1	Isolated from the spontaneous wine fermentation at the Yalumba Wine Company (Angaston, Australia)	CP027647.1 to CP027655.1	Whole-genome map	[17]
NRRL Y-50541	Isolated from the mezcal-fermenting process at Oaxaca, Mexico	CP011778 to CP011785	Chromosome	[16, 18]
SRCM101298	Isolated from fermented food by Sung Ho Cho, Microbial Institute for Fermentation Industry, South Korea	GCA_002214845.1	Contig	[19]

Table 1.
Genomes sequencing projects developed for Torulaspora delbrueckii.

been recently assembled, having been isolated from a spontaneous wine fermentation from Australia. Its genome consists of eight chromosomes and one mitochondrial chromosome. Altogether, these published data sets are proving to be useful as they guide the design of experimental approaches to study enzymes involved in the biosynthesis and/or the catabolism of biotechnological molecules of interest.

The discovery of the circular plasmid pTD1 from *T. delbrueckii* CBS 1090 provided an excellent platform for the development of modern genetic tools. The circular pTD1 (4.8 kbp) is an unusual finding in the diverse yeast genera that normally possess linear plasmids [20]. The plasmid pTD1 belongs to the 2-μm family, and it has been shown that the members of this plasmid family keep a high-copy number with minimal impact on the host. Recently, a model was suggested for the 2-μm protein complexes required for plasmid partitioning and transcriptional regulation [21]. pTD1 also contains an AT-hook motifs (residues 222–234) which are small DNA-binding modules with a preference for binding AT-rich DNA [22] and have been frequently identified in a large number of proteins, such as transcription and chromatin remodelling factors, such as the yeast centromere-binding protein and Mif2 DNA replication origin recognition factor Orc2, respectively [21, 23]. Two decades after the discovery of pTD1, its importance has been recently brought to light when a modified version for the TD recombinase (variant TD1–40) was employed to the development and analysis of two Flp-like site-specific recombination systems. The evolved variants of the TD recombinase, unlike the wild-type enzyme, were suitable for genome engineering in *Escherichia coli* and mammalian cells [24]. The authors also suggest that its application could be potentially extended to plant and insect cells.

The allele-coupled exchange (ACE) and the allelic exchange (AE) technologies are becoming more common for the manipulation of microorganisms for biotech purposes. For example, researchers lead by Professor Nigel Minton at the Synthetic Biology Research Centre (SBRC) (University of Nottingham, UK) have successfully dedicated their efforts to develop the ACE and the AE technologies in diverse *Clostridia* strains and other Gram-positive bacteria [25, 26]. The genetic manipulation of *T. delbrueckii* was initially achieved using the genetic tools developed originally for *S. cerevisiae.* The improved gene disruption method for *T. delbrueckii* was performed using the classic PCR-based disruption cassettes, one of the most commonly used strategies for gene targeting in *S. cerevisiae* and other organisms [12]. Eventually, ACE was adapted for baker's yeast *T. delbrueckii* IGC5323 and was one of the goals for the development of genetic tools towards the metabolic engineering of *T. delbrueckii* [*27*].

At the present time, many attempts have been made to expand the number of selection markers available for the construction of new molecular tools for *T. delbrueckii*; however, the pursuit of selection markers remains unsuccessful [12]. A successful auxotrophic selection marker was the creation of a screening system under glucose as sole carbon source. The deletion of LGT1 gene, identified as coding for a hexose transporter, was successfully conducted, despite the fact that the deletion did not impaired glucose uptake ability [28]. According to previous works, it is likely that the genome of *T. delbrueckii* contains other hexose transporters [29]. Other approaches are still being developed towards genome editing, and trends indicate that eventually the modern CRISPR-CAS9 technique could be adapted for *T. delbrueckii*, just as it was recently fully adapted for *S. cerevisiae* [30].

3. From the lab to the kitchen: efforts towards the incorporation of scientific research into the baking and brewing industries

Results derived from scientific research have positively influenced many aspects of human wellbeing, such as food production. Many food industries are currently

interested in innovation through the usage of *T. delbrueckii* strains in their fermentative processes. Consequently, the brewing and baking industries are starting to use nonconventional yeast strains in their processes.

Effective biomass production from molasses is a crucial aspect to consider in the selection of baker's yeast for the industrial process. Baker's yeast is able to propagate and to create biomass from sugarcane molasses, which contains mainly glucose, fructose, and sucrose as well as trisaccharide raffinose [31, 32]. On the other hand, invertase activity is crucial for the hydrolysis of disaccharide into free glucose and fructose monomers, required for yeast growing on molasses.

Yeast strains with high invertase activity exhibit a low production of CO_2 in the sweet dough. This last observation can be explained by the rapid entry of free sugars into the cells causing a massive increase in the osmotic pressure that affects the cellular homeostasis which eventually slows glycolysis [33–35].

Moreover, both trehalose and glycogen are known as the major store of glucose into *Saccharomyces cerevisiae* cells. A rapid increase of the levels of these sugars is an early metabolic response during conditions of oxidative, heat, or salt/osmotic stresses. It has been proposed that these sugars have several physiological functions. For example, the accumulation of these sugars has a function as glycolytic safety valves to escape "substrate-accelerated death": a phenomenon that follows when the starved yeast is exposed to the substrate, which limits their growth [36–39].

Oppositely, *Torulaspora delbrueckii* strains under hyperosmotic/frozen stresses into sweet dough have shown the ability to adapt promptly to high-osmotic-pressure environments which correlates in part with a low-invertase activity, as well as a slow rate of trehalose mobilisation, displaying a higher accumulation of trehalose than *S. cerevisiae* [8, 27]. Moreover, the intracellular trehalose accumulation, induced by desiccation of *T. delbrueckii*, protects the yeast cell and presents a lower oxidative stress, which correlates with a higher fermentative capacity, when compared to other nonconventional yeasts [40]. The trehalose content may possibly be correlated to their types of trehalase. Strain D2–4 was found to harbour two types of trehalase activities, which have different optimum reaction pH at 4.3 and 6.7. On the other hand, the freeze-sensitive mutant strain 60B3 bears additional activity at pH 5.7, which adds a third type of trehalase activity in that sensitive mutant. The change of trehalose content during the growth of the freeze-sensitive 60B3 strain was directly correlated with the change of the trehalase activity at pH 5.7. Despite the fact that higher trehalose levels are always correlated with higher stress resistance, experimental results suggest that other factors are involved in the maintenance of stress resistance [41–43].

As mentioned above, the metabolism of *T. delbrueckii* and *S. cerevisiae* differs greatly, such as their regulatory mechanisms in the adaptive response to salt stress [44]. The gene ENA1, encoding a Na^+-ATPase, is a key determinant in the *Saccharomyces cerevisiae* tolerance when it is exposed to salt stress as high concentrations of extracellular toxic monovalent cations (i.e. Na^+ and Li^+) [45]. The regulation of ENA1 required for cell survival is mainly under the control of three diverse signalling pathways, (1) high-osmolarity glycerol (HOG), (2) cell wall integrity (CWI), and (3) calcineurin/Crz1p pathways [46–48]. Glycerol production is essential for *S. cerevisiae* to deal with osmotic stresses. Hog1 phosphorylation occurs as a response to osmotic stress, and the phosphorylated Hog1 interacts with transcription factors to modulate its gene expression patterns during stress responses [49, 50].

In order to understand the high-osmolarity glycerol (HOG) pathway in *T. delbrueckii*, the Hog1 homologue in *T. delbrueckii* (*TdHog1*) was characterised as well as its mutant versions. The expression of GPD1 was not strongly affected in the *TdHog1*-deleted mutant. Also, *S. cerevisiae* and *T. delbrueckii* have divergent regulatory mechanisms that control glycerol accumulation under a moderate osmotic stress condition [51]. As a consequence of glycerol accumulation, in anaerobiosis,

glycerol production of *T. delbrueckii* represents around 6% of the total carbon flux, a value that is comparable to *S. cerevisiae* under the same conditions. However, acetic acid levels are increased which allows for reduction of NAD+ generated during glycerol formation in *S. cerevisiae* as a side-effect of the overexpression of GPD1 and/or GPD2 in the presence of high-glycerol concentration. A significant relationship between glycerol and acetic acid production was not found in *Torulaspora delbrueckii* [52, 53]. When an excess of acetic acid is produced in alcoholic beverages such as a beer or wine, it provides a characteristic vinegar-like flavour or sour taste [54, 55]. A higher percentage of acetic acid also results in decreased CO2 production [9, 56–58].

Saccharomyces cerevisiae exhibits an adaptive response to cell wall stress mediated by cell wall integrity MAPK signalling factor Slt2, which once activated triggers Rlm1 phosphorylation [59]. Additionally, the *slt2*Δ mutant allowed for the discovery of a novel regulatory mechanism associated with PKA inhibition [60, 61]. During the study of *hog1* mutant from *S. cerevisiae*, it has been reported that Slt2p is rapidly dephosphorylated (1 min). On the other hand, a Tdhog1 mutant from *T. delbrueckii* showed a significant reduction of the phospho-Slt2p signal, and it was delayed around 30–60 min [51].

The induction of the ENA1 gene is mediated by calcineurin which plays a role in the dephosphorylation of Crz1. Two Crz1-binding regions have been identified into the ENA1 promoter; the antagonistic regulation of ENA1 is via protein kinase A (PKA) that is involved in the Crz1 phosphorylation [62]. Additionally, phosphatases Ppz1 and Ppz2, individually or both, influenced the expression of ENA1. For example, a higher increase of the ENA1 expression in Ppz1 mutants was observed which was correlated to an increase halotolerance. Interestingly, Ppz1 showed to be dependent of an intact calcineurin/Crz1-signalling pathway to keep its ENA1 promoter activity [63]. During the analysis of TdCRZ1, the homologue to *CRZ1* in *Torulaspora delbrueckii*, the authors tried to enhance the salt tolerance of *S. cerevisiae* HS13. So, the TdCrz1p was linked to the freeze tolerance capability, a very important feature with great potential for industrial applications [44].

4. Yeast cocultures: *Torulaspora delbrueckii*—combining to enhance the product quality

At the present time, commercial strains of *Torulaspora delbrueckii* are used in the bread and brewing industries and are summarised in **Table 2**. The success of *T. delbrueckii* relies mainly on the superior flavour that its fermentation can provide to the final product and also in its incredible ability to withstand stress conditions described previously. These properties will lead to improve the product quality and enhance the flavour complexity. However, there are still some challenges to overcome to employ a pure culture of *T. delbrueckii* in industrial fermentations.

Name	Supplier	Industry		Reference
		Bakery	Brewery	
Biodiva™TD291	Lallemand		✓	[64]
ZYMAFLORE® ALPHA*TD n. sacch*	Laffort		✓	[10]
H299–18	Nikka Uisukii KK	✓		[65]
NS8422 (FERM P-12709)	Nikka Whiskey KK	✓		[66]

Table 2.
A brief list of Torulaspora delbrueckii commercially available.

For this reason, these yeast strains are usually employed in co-fermentations with *Saccharomyces cerevisiae* and/or other yeast strains [9, 10]. For example, in mixed fermentations, it plays an important role to enhance its organoleptic properties such as flavour, aroma, and a final product with low-alcohol content [9].

The current key market players for global yeast market are listed below:

- AB Mauri
- Biospringer
- Chr. Hansen Holding A/S
- Lesaffre
- AB Vista
- Alltech
- Angel Yeast Co., Ltd.
- Biorigin—Art in Natural Ingredients
- DSM N.V.
- ICC
- Kerry Group
- Lallemand Inc.
- Leiber GmbH
- Minn-Dak Yeast Company
- Ohly
- Oriental Yeast Co., Ltd.
- Pacific Ethanol, Inc.
- Pakmaya
- Suboneyo Chem. Pharmaceuticals Pvt. Lim.
- Synergy Flavours

The flavour profiles of co-fermentations in brewing showed significant differences, revealing a species-dependent relationship. Analysis of the main volatile compounds on the beers produced by *T. delbrueckii* and *S. cerevisiae* in the pure cultures and their simultaneous co-fermentation at different ratios was performed. *T. delbrueckii* was able to increase the levels of higher alcohols, where β-phenyl ethanol was excluded. These results are opposite to the data obtained in winemaking, where lower levels of higher alcohols are detected. β-phenyl ethanol and ethyl

butyrate levels were lowered when a major presence of *T. delbrueckii* was present in the inoculum ratio [9–11]. A yeast population change has been correlated with the inoculum size ratio and the type of cell-to-cell interactions that occur during mixed culture fermentation. The faster decay of *T. delbrueckii* during cocultures with *S. cerevisiae* is notable below a 2:1 ratio. The last observation could be related to multiple factors, for example, due to the *T. delbrueckii*'s killer activity: a kind of immune system that secretes protein toxins, which become lethal to other yeast species [67, 68].

Sequential inoculation of *T. delbrueckii* with *S. cerevisiae* has shown higher levels of desired aroma compounds compared to simultaneous inoculation, exemplified by the enhanced production of 4-vinyl guaiacol (clove-like aroma) [69].

Unlike the production of beer and wine, co-fermentation with *T. delbrueckii* during dough preparation has been barely explored; only simultaneous co-fermentations have been performed. *T. delbrueckii* JK08 and *P. anomala* JK04 exhibited a superior flavour and mouthfeel, respectively, as well as an improvement in the colour, when compared to *S. cerevisiae*. Even when these strains did not fulfil the exact criteria of commercial baker's yeasts [70], the use of these three yeasts together has been proven to enhance the bread quality [70].

5. Conclusion

The history of fermented foods has long accompanied the evolution of the human civilisation. While the ingredients may have changed, their selection has also been shaped to meet new consumer demands. In fact, over centuries, man has refined the art of fermented food by pure empirical observation, for example, through the selection of the best cereals with better performing characteristics or through the domestication of yeast strains. Despite the fact that the fermented food industry has mastered the domestication of *Saccharomyces cerevisiae*, the use of nonconventional yeast strains has proved to be advantageous for the fermented food industry.

Once considered a microbial contamination, *Torulaspora delbrueckii* has been shown to have a positive influence on several organoleptic properties for the final products of the brewing and bread industries. For example, when this nonconventional yeast is used in the brewing industry, the aromatic profiles and flavours of the beer are enhanced; and some strains have the peculiarity to produce naturally less-ethanol content while retaining all its improved properties. Additionally, many of its other biological features were conveniently suitable for the industrial processes, such as its robustness potential to resist frozen/thaw stress. These advantages will be useful for biotechnological exploitation that will lead to innovation of new products or even to process improvement for the food industry. As usual in new trends in science, to date, the information regarding *T. delbrueckii* genetics and its metabolic pathways remain scarce. Fortunately, the scientific community is starting to focus their efforts to deeply understand the nature of this splendid genus. Continued scientific research will gradually lead to the accumulation of more data that will impact positively the fermented food industry.

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Conflict of interest

The authors declare that they no conflicts of interest.

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From time immemorial fermented foods have undoubtedly contributed to the progress of modern societies. Historically, ferments have been present in virtually all human cultures worldwide, and nowadays natives from many ancient cultures still conduct a wide variety of food fermentations using deep-rooted recipes and processes. Within the last four centuries, scientific research has started to unravel many aspects of the biological process behind fermentations, which has contributed to the improvement of many industrial processes. During our journey in the research field, we have always been attracted to the development of scientific research around fermentations, especially autochthonous ferments: a natural repository of novel biomolecules and biological processes that will positively impact on many application fields from health, to food, to materials.

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