Understanding Kombucha Tea Fermentation: A Review

Silvia Alejandra Villarreal-Soto, Sandra Beaufort, Jalloul Bouajila, Jean-Pierre Souchard, and Patricia Taillandier

Abstract: Kombucha is a beverage of probable Manchurian origins obtained from fermented tea by a microbial consortium composed of several bacteria and yeasts. This mixed consortium forms a powerful symbiosis capable of inhibiting the growth of potentially contaminating bacteria. The fermentation process also leads to the formation of a polymeric cellulose pellicle due to the activity of certain strains of *Acetobacter sp*. The tea fermentation process by the microbial consortium was able to show an increase in certain biological activities which have been already studied; however, little information is available on the characterization of its active components and their evolution during fermentation. Studies have also reported that the use of infusions from other plants may be a promising alternative.

Keywords: bioactivity, fermentation, kombucha tea, microbial cellulose, symbiosis

Practical Application: Kombucha is a traditional fermented tea whose consumption has increased in the recent years due to its multiple functional properties such as anti-inflammatory potential and antioxidant activity. The microbiological composition of this beverage is quite complex and still more research is needed in order to fully understand its behavior. This study comprises the chemical and microbiological composition of the tea and the main factors that may affect its production.

Introduction

Fermentation is one of the most antique methods of food preservation. It is also a low-cost energy conservation system, which is essential to ensure the life and safety of food. Many biochemical changes occur during fermentation and may affect the nutrient compounds and consequently the properties of the final product, like the bioactivity and digestibility. Recently, this bioprocess has been applied for the production and extraction of bioactive compounds from plants in food and beverage industries (Hur, Lee, Kim, Choi, & Kim, 2014).

Kombucha tea is obtained from a symbiotic culture of acetic acid bacteria (AAB; *Komagataeibacter*, *Gluconobacter*, and *Acetobacter* species) (Roos & Vuyst, 2018), lactic acid bacteria (LAB; *Lactobacillus*, *Lactococcus*) (Marsh, Hill, Ross, & Cotter, 2014), and yeasts *(Schizosaccharomyces pombe*, *Saccharomycodes ludwigii*, *Kloeckera apiculata*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Torulaspora delbrueckii*, *Brettanomyces bruxellensis*) (Coton et al., 2017) in a sweet medium, generally black tea. Its fermentation process also leads to the formation of a floating biofilm on the surface of the growth medium due to the activity of certain strains of AAB (Watawana, Jayawardena, Gunawardhana, & Waisundara, 2016). The main acids present are acetic, gluconic, tartaric, malic, and in less proportion citric acid. All these acids are responsible for its characteristic sour taste (Jayabalan, Marimuthu, & Swaminathan, 2007). Actual food trends toward minimally processed products, without additives, high nutritional value and with health benefits have increased with consumer awareness. In this context, the traditional Kombucha tea has recently captured the attention of researchers and consumers because of its probiotic characteristics. However, the manufacturing technology, its microbiota, byproducts, and physicochemical properties are important facts to consider for industrial production. There are several types of fermentation and obtained products depending on the metabolic pathway followed. Kombucha fermentation is a combination of three of them: alcoholic, lactic, and acetic one, this because of the presence of several yeasts and bacteria coexisting in the medium. Being initiated by osmotolerant microorganisms and ultimately dominated by acid-tolerant species. Several authors have studied the benefits of Kombucha tea; however, there is little information on the characterization of its active components, their evolution during fermentation, and their pharmacological activities. Moreover, the influence of fermenters, substrates, metabolites, and their improvements on the organoleptic qualities and fermentation kinetics should be also evaluated.

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Kombucha Tea: A Complex Fermented Beverage

Kombucha is a popular drink among many traditional fermented foods. Bacteria and yeasts present in the medium create a powerful symbiosis capable of inhibiting the growth of contaminating microorganisms (Vitas, Malbasa, Grahovac, & Loncar, 2013). It is composed of two phases: a floating biofilm and a sour liquid phase. Acetic acid, gluconic acid, and ethanol are the main components in the liquid, but also in the biofilm due to its great water absorption capacity (Czaja, Krystynowicz, Bielecki, & Brown, 2006). Under aerobic conditions the symbiotic consortium of Kombucha is able to convert sugar and tea in a period from 7 to 10 days in a lightly carbonated, slightly sour, and refreshing drink, which is composed of several acids, 14 amino acids, vitamins, and some hydrolytic enzymes (Malbaša, Lončar, Vitas, & Čanadanović-Brunet, 2011).

Chemical composition

Detailed knowledge of the composition and properties of Kombucha tea is crucial for better understanding its kinetics. However, the composition and metabolite concentration are dependent on

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Table 1–General chemical composition of Kombucha.

	Compound	Average composition	Initial sucrose	Fermentation time (days)	References
Organic acids	Acetic acid	5.6 g/L	70 g/L	15 d	Blanc (1996)
	Acetic acid	8.36 g/L	100 g/L	18 d	Jayabalan et al. (2007)
	Acetic acid	11 g/L	100 g/L	30 d	Chen and Liu (2000)
	Gluconic acid	39 g/L	100 g/L	60 d	Chen and Liu (2000)
	Glucuronic acid	0.0160 g/L	70 g/L	21 d	Lončar et al. (2006)
	Lactic acid	0.18 g/L	100 g/L	18 d	Jayabalan et al. (2007)
Vitamins	Vitamin B1	0.74 mg/mL	70 g/L	15 d	Bauer-Petrovska and Petrushevska-Tozi (2000)
	Vitamin B2	8 mg/100 mL	70 g/L	10 d	
	Vitamin B6	0.52 mg/mL	70 g/L	15 d	Malbaša et al. (2011)
	Vitamin B12	0.84 mg/mL	70 g/L	15 d	Bauer-Petrovska and Petrushevska-Tozi (2000)
	Vitamin C	25 mg/L	70 g/L	10 d	Bauer-Petrovska and Petrushevska-Tozi (2000) Malbaša et al. (2011)
General composites	Ethanol	5.5 g/L	100 g/L	20 d	Chen and Liu (2000)
	Proteins	3 mg/mL	100 g/L	12 d	Jayabalan et al. (2007)
	Tea polyphenols	7.8 Mm GAE	100 g/L	15 d	Chu and Chen (2006)
Minerals	Cu, Fe, Mn, Ni, Zn	0.1 to 0.4 μg/mL	70 g/L	15 d	Bauer-Petrovska and Petrushevska-Tozi (2000)
Anions	F^-, Cl^-, Br^-, I^-, NO_3^-, HPO_4^- SO_4^-	0.04 to 3.20 mg/g	100 g/L	7 d	Kumar, Narayan, and Hassarajani (2008)

the inoculum source (Nguyen, Nguyen, Nguyen, & Le, 2015), the sugar and tea concentration (Fu, Yan, Cao, Xie, & Lin, 2014; Watawana and others 2017), the fermentation time (Chen & Liu, 2000), and the temperature used (Jayabalan et al., 2008; Lončar, Djurić, Malbaša, Kolarov, & Klašnja, 2006). Any change in the fermentation conditions might affect the final product. Nevertheless, the main components and some key metabolites produced in the fermented tea are present below in Table 1.

Final sugar concentrations can differ from one fermentation to another, which indicates that the metabolic pathway does not always occur in the same way (Chen & Liu, 2000). Regarding the production of organic acids, Jayabalan et al. (2007) observed the increasing production of acetic acid through the fermentation until a maximum of 9.5 g/L after 15 days. In the case of D-glucuronic acid, it reached a maximum concentration of 2.3 g/L on the 12th day and in less quantity 0.54 g/L of lactic acid was detected in the 3rd day. As for the anionic concentration, it remains at low values, ranging between 0.04 and 3.20 mg/g, being F^- and Cl^- the most present anions (Watawana, Jayawardena, Gunawardhana, & Waisundara, 2015). The chemical composition as well as the concentration of each metabolite produced in Kombucha will always depend on the inoculum, initial sugar concentration, and so on. However, among the main constituents of Kombucha tea, glucuronic acid is considered to be the main therapeutic agent (Kumar & Joshi, 2016).

Microbiological composition

Several studies have shown that the microbial spectrum of Kombucha consortium, also called SCOBY or tea fungus, may vary between fermentations (Chakravorty et al., 2016; Coton et al., 2017; Reva et al., 2015). However, there is a number of species that remains in most of Kombucha cultures, which are described next.

Yeasts

Most yeasts species can ferment sugar to ethanol, yet many modern alcoholic fermentation processes are initiated by a single starter culture, commonly being *Saccharomyces cerevisiae* due to its high efficiency. However non-*Saccharomyces* yeasts are becoming increasingly used in the industry in mixed fermentations (wine, tequila, and so on) in order to enrich the aromatic profile, and to enhance the complexity and the kinetics of the final product (Lopez, Beaufort, Brandam, & Taillandier, 2014; Nehme, Mathieu, & Taillandier, 2008). Microbial interactions between *Saccharomyces* and non-*Saccharomyces* yeasts seems to be an advantageous option in mixed fermentation processing, having several benefits like avoiding the risks of stuck fermentation, the addition of aromas and flavors, allows the modification of undesired parameters, between others (Sun, Gong, Jiang, & Zhao, 2014). And in this sense, Kombucha's yeasts interaction has proven to be a consortium that generates final desirable characteristics.

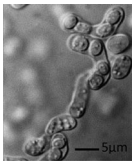
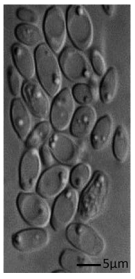
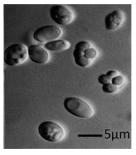
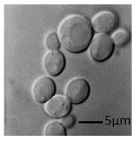
There are many yeasts genus and species in Kombucha culture, a broad spectrum has been reported including species of *Zygosaccharomyces*, *Candida, Kloeckera/Hanseniaspora*, *Torulaspora*, *Pichia*, *Brettanomyces/Dekkera*, *Saccharomyces*, *Lachancea*, *Saccharomycoides*, *Schizosaccharomyces*, and *Kluyveromyces* (Chakravorty et al., 2016; Coton et al., 2017; Marsh et al., 2014). Despite of its variability, some of the predominant species are presented in Table 2.

In addition to those already mentioned, several authors have quantified some other yeasts present in the Kombucha culture, Watawana et al. (2016) reported *Zygosaccharomyces* as the predominant yeast with 84.1% of relative percentage of abundance and *Dekkera* and *Pichia* species with 6% and 5%, respectively. Mayser (1995), revealed biofilm-forming yeasts such as *Candida krusei* or *Issatchenkiaorientalis* as well as species of the apiculatus yeasts (*Kloeckera*, *Hanseniaspora*). A new ascosporogenous yeast called *Zygosaccharomyces kombuchaensis* was isolated from Kombucha tea by Kurtzman, Robnett, and Basehoar-Powers (2001).

Bacteria

The dominant bacteria of Kombucha tea culture are AAB, which are aerobic bacteria able to use alcohol as a substrate to form acetic acid. These bacteria, in contrast to yeast, require large amounts of oxygen for their growth and activity. The metabolic process is based on the conversion of acetaldehyde into ethanol and acetaldehyde hydrate into acetic acid by the enzyme

Table 2–Some yeasts species present in Kombucha culture.

Species	Morphology	Characteristics
Schizosaccharomyces pombe		• High fermentative power • Ability to convert malic acid to ethanol • Release of high quantities of polysaccharides (Domizio, Liu, Bisson L, & Barile, 2017)
Brettanomyces bruxellensis		• High resistance to osmotic and ethanol stress • Higher efficiency to utilize the available N sources than *Saccharomyces cerevisiae* • Tendency to ferment sugars to ethanol, and produce high concentrations of acetic acid in aerobic conditions (Steensels et al., 2015)
Saccharomyces cerevisiae		• High ethanol tolerance • Rapid fermentation rates • Insensitivity to temperature and substrate concentration (Choonut, Saejong, & Sangkharak, 2014)
Zygosaccharomyces rouxii		• Highly osmo- and halo-tolerant • Counteract better sugar and salt stress than *S. cerevisiae* (Dakal et al., 2014)

Only images from Pitt and Hocking (2009) with modifications.

acetaldehyde dehydrogenase (Jayabalan et al., 2007). Several AAB are present in the tea fungus, including: *Acetobacter xylinoides*, *Bacterium gluconicum*, *Acetobacter aceti*, *Acetobacter pasteurianus*, and *Gluconobacter oxydans* (Jayabalan, Malbaša, Lončar, Vitas, & Sathishkumar, 2014). Marsh et al. (2014) worked with rRNA sequence analysis and found between 86% and 99% relative abundance of *Gluconacetobacter* through all the fermentation both in the liquid medium and in the biofilm. Similar results were obtained by Watawana et al. (2016) who fermented coconut water with the tea fungus and found *Gluconacetobacter* as the main one with a relative percentage of 85.6 and in less proportion *Acetobacter*, *Lactobacillus*, *Leuconostoc*, and *Bifidobacterium* species.

Cellulose production

There are several types of bacteria that can produce microbial cellulose, such as: *Aerobacter*, *Agrobacterium*, *Azotobacter*, *Rhizobium*, *Salmonella*, and *Gluconacetobacter* (Mohite & Patil, 2014). Among the *Acetobacter* gender, the dominant specie is *Acetobacter xylinum*, which was reclassified as *Gluconacetobacter xylinus* and more recently to *Komagataeibacter xylinus* (Yamada et al., 2012). A specific biochemical activity of this bacterium is the oxidation of glucose to gluconic acid, which is found in the liquid phase, then another specific metabolism leads to the synthesis of microbial cellulose forming the biofilm that remains in the liquid surface. The process includes the synthesis of uridine diphospho-glucose (UDPGlc) (Figure 1), which is the cellulose precursor, then each single cell of *Acetobacter* may polymerize up to 200,000 glucose residues per second into β-1,4-glucan chains. The advantage of this form of cellulose production is that the bacterium grows rapidly under controlled conditions and can produce cellulose from a variety of carbon sources including glucose, ethanol, sucrose, and glycerol.

The microbial cellulose is produced extracellularly in the form of fibrils that are attached to the bacterial cell. Each single cell has between 50 and 80 pores or complex terminals (CTs) with a diameter of 3.5nm for extruding cellulose out of their membrane (Figure 2). These chains are later assembled forming thicker fibrils called macrofibrils creating a 3-D structure of about 1,000 individual glucan chains which can hold up to 200 times more water of its dry mass and possess high conformability and great elasticity. Bacteria produce two forms of cellulose, cellulose I and cellulose II. Cellulose I is a ribbon-like polymer composed of bundles of microfibrils, while cellulose II is an amorphous polymer that is thermodynamically more stable than cellulose I (Podolich et al., 2016).

In the first state, the producing cellulose bacteria increase its population through the consumption of the dissolved oxygen. During this time, the microorganism synthesizes certain amount of cellulose in the liquid medium and just the bacteria that are in the air/medium interface can maintain its activity and produce cellulose, which is formed by superimposed layers. As fermentation time advances, the membrane thickness is increased by the generation of new layers on the surface, forming a suspended structure in the culture medium. The development of the biofilm along with hydrogen and C-H bonding will continue through all fermentation, its synthesis will reach its limit when it grows downward entrapping all bacteria, which then will become inactive due to insufficient oxygen supply (Esa, Tasirin, & Rahman, 2014). The bacteria remaining in the liquid phase of the culture medium are in a dormant state and can be reactivated and used as inoculum in a later fermentation (Ruka, Simon, & Dean, 2012). This biofilm possesses high crystallinity, high tensile strength, extreme insolubility in most of the solvents, moldability, high degree of polymerization, it is 100 times thinner than that of cellulose fibrils obtained from plants, and its water holding capacity is over 100 times higher (Chawla, Bajaj, Survase, & Singhal, 2009). One of its main characteristics is its purity, which distinguish it from the plant one that contains hemicellulose and lignin (Sulaeva, Henniges, Rosenau, & Potthast, 2015), but also its high degree of crystallinity (>60%) where the crystals formed are composed of cellulose type Iα and Iβ. These unique properties, as well as its purity have allowed many successful applications in the field of biomedical materials (Kuo, Chen, Liou, & Lee, 2015). The biofilm may vary depending on the used strains, culture time, and chemical additives present in the culture media (Lee, Gu, Kafle, Catchmark, & Kim, 2015), but it is also hypothesized that microbial cellulose obtained from a Kombucha culture may produce a biofilm with different characteristics than those form typical sources (Nguyen, Flanagan, Gidley, & Dykes, 2008). There are several factors to consider in order to maximize the yield of microbial cellulose and optimize the process, for example, the volume of the inoculated media, the incubation time, the surface area, and surface height conditions (Cacicedo et al., 2015). The removal of the amorphous parts of the nanofibrils through acid hydrolysis can produce nanocrystals, which can be used for different purposes in several areas as food packaging or medical applications (Campano, Balea, Blanco, & Negro, 2016).

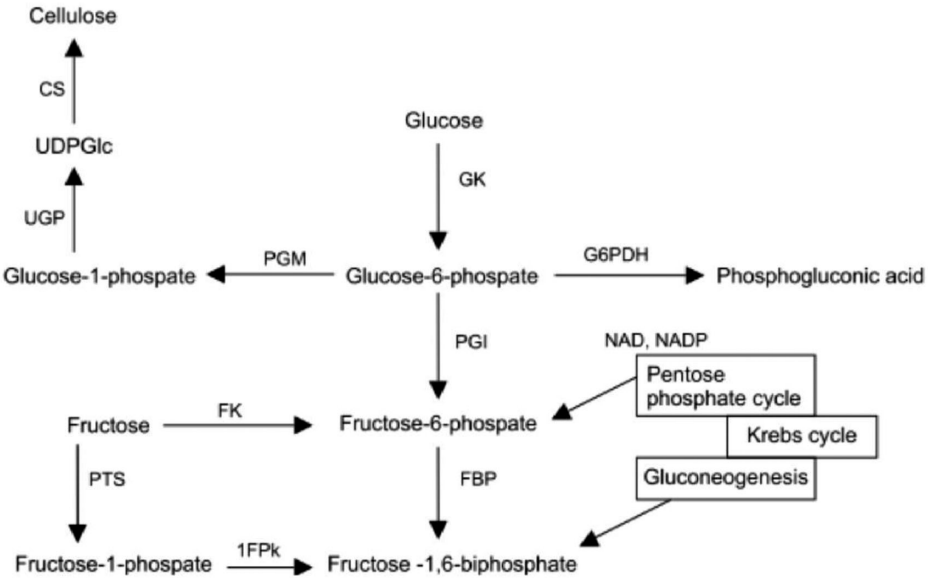


Figure 1–Biochemical pathway for cellulose synthesis by *A. xylinum*. CS, cellulose synthase, GK, glucokinase, FBP, fructose-1,6-biphosphate phosphatase, FK, fructokinase, 1FPk, fructose-1-phosphate kinase, PGI, phosphoglucoisomerase, PMG, phosphoglucomutase, PTS, system of phosphotransferases, UGP, pyrophosphorylase uridine diphosphoglucose, UDPG, lcuridine diphosphoglucose, G6PDH, glucose-6-phosphate dehydrogenase, NAD, nicotinamide adenine dinucleotide, NADP, nicotinamide adenine dinucleotide phosphate (Chawla et al., 2009).

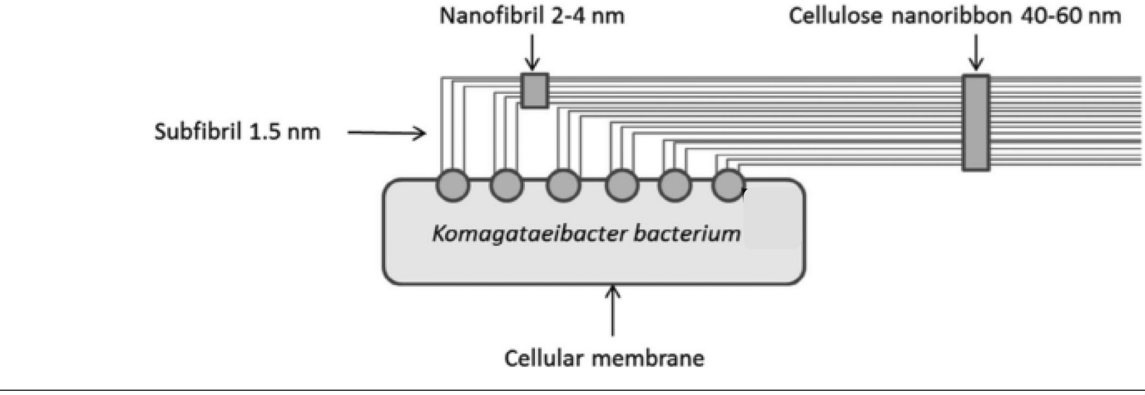


Figure 2–Assembly of cellulose microfibrils by *K. xylinum* (Cacicedo et al., 2015, after modifications).

Microorganisms Interactions

The complexity of understanding Kombucha fermentation kinetics are mainly due to the important number of microorganisms present and the interactions between them (Markov, Jerinic, Cvetkovic, Loncar, & Malbasa, 2003), which are considered to have inhibitory effects on the ethanol production. However, the death and autolysis of yeast cells releases also vitamins and other nutrients that stimulate the growth of important bacteria. Most microbial species excrete metabolic products that can either stimulate or inhibit the specific growth rate of the other species, establishing commensalistic or amensalistic interactions which have to be extensively analyzed to achieve the comprehension of this phenomenon of coexistence. Some bacteria groups such as LAB and AAB, as well as yeasts species such as *Saccharomyces cerevisiae*, have well-established roles in the fermentation. However, until today, there are many other species whose roles have not been extensively characterized, nor the interactions between them. There are a number of obstacles in understanding microbial ecosystems, the first one is the enormous diversity and complexity of most of the microbial communities, for example, certain microorganisms can participate in parallel, while others act in a sequential manner with a dominant evolution during fermentation (Chakravorty et al., 2016). In the case of Kombucha, the different yeasts and bacteria species act in parallel producing two different final products: the fermented tea and the biofilm. At the beginning of the fermentation, yeast hydrolyze sucrose into glucose and fructose, formerly the ethanol is produced and finally AAB transform ethanol into acetic acid, nonetheless the production of gluconic and glucuronic acids is also remarkable (Figure 3).

Microbial identification methodologies

Traditionally, microorganisms have been classified and identified mainly by morphological and physiological criteria, nevertheless in addition to these standard tests, biochemical methods provide important data for the characterization, however, the result of these tests sometimes lead to erroneous characterizations because these functions are controlled by one or few genes. Besides, all these tests take time and sometimes their determination and classification is ambiguous due to the variability of the species. That is why, it is recommended to complement the conventional techniques with molecular techniques to elucidate not only the

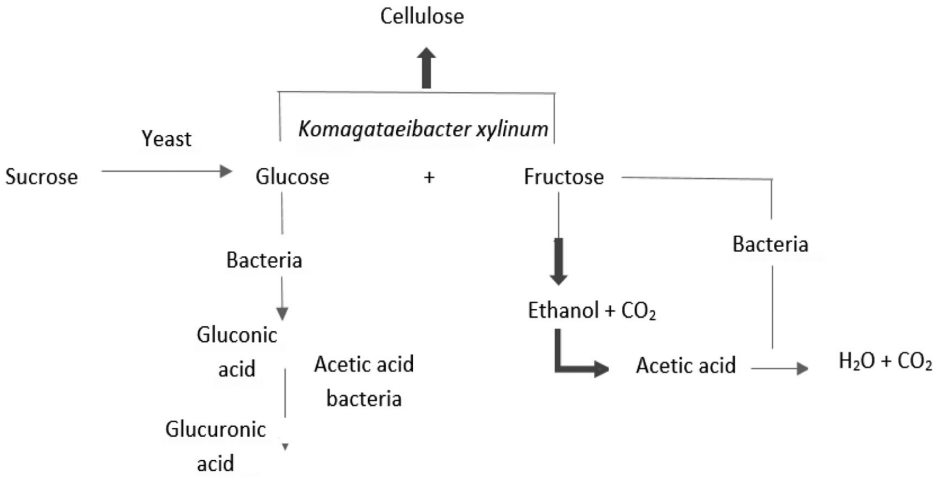


Figure 3–Main metabolic activity of Kombucha tea (Markov et al., 2003).

degree of relationship, but also to reveal the connections between evolutionary mechanisms (Kurtzman et al., 2001).

Chen and Liu, (2000) reported a technique for enumeration of AAB and yeast in the liquid and in the biofilm. They declared the number of viable cells and were also able to isolate some of the yeasts and main AAB throughout the fermentation, where they noted that the number of viable cells was greater in the liquid broth than in the biofilm. The protocol was based on the use of potato dextrose and GYCA agar medium to numerate yeasts and AAB from 20 g of sample homogenized in a blender for 9 min then incubated at 30 °C for 3 days. Cell counts were expressed as colony-forming units per milliliter. Hidalgo, Mateo, Mas, and Torija (2012) worked with yeasts and bacteria identification methods and obtained 453 yeasts and 270 bacteria isolates. In the case of yeasts, YPD agar was used (yeast extract 10 g/L, bacteriological peptone 20 g/L, D-glucose 20 g/L, and agar 20 g/L) and incubated for 48 hr at 28 °C. Then about 25 and 30 colonies were randomly picked and plated on a selective lysine medium to differentiate *Saccharomyces* and non-*Saccharomyces* yeasts, and finally a PCR reaction mix and RFLP- PCR of rDNA was performed to identify the yeasts species. For the bacteria isolation method, GYCA medium was used (10% glucose, 1% yeast extract, 2% $CaCO_3$, and 1.5% agar supplemented with 100 mg/L of natamicine). Then total DNA was extracted using the CTAB method (cetyltrimethylammonium bromide), all AAB were genotyped using ERIC and (GTG) 5-rep-PCR fingerprinting techniques, and the electrophoresis was analyzed in 1.5% (w/v) agarose gels. Colonies with a halo around them were subjected to Gram staining and catalase test, which verified their identity as AAB. Enterobacterial Repetitive Intergenic Consensus-PCR and Repetitive Palindromic-PCR have been proposed as suitably accurate techniques for identifying bacteria strains and for determining taxonomic relationships between bacterial species because of their high degree of polymorphism.

It is recommended to use combined techniques such as RFLP because it seems to provide additional information to establish a phylogenetic placement of several real yeasts. This was demonstrated by Kurtzman et al. (2001), where the combination of these techniques allowed to distinguish the different species within the genus *Zygosaccharomyces*, obtained from Kombucha isolates. According to Meersman et al. (2013), the use of a traditional method combined with one of accurate identification such as ribosomal DNA, sequence-based PCR, or fragment length polymorphism will lead to a more precise quantification of the microbiota and a further isolation of potential starter cultures. However, the first culture-independent, high-throughput sequencing analysis of the Kombucha biofilm was done by Marsh et al. (2014) with which they were able to identify the major bacterial and yeast populations present both in the biofilm and in the liquid broth, being *Gluconacetobacter* and *Zygosaccharomyces*, respectively.

Factors Influencing Kombucha Fermentation

Fermentation is influenced by many factors such as temperature, pH, the amount of oxygen, the CO_2 dissolved, the operating system, the supply of precursors, the shear rate in the fermenter, as well as the nature and composition of the medium (Marsh et al., 2014). Any variation in these factors can affect the rate of fermentation, the spectrum, the performance, the organoleptic properties, the nutritional quality, and other physicochemical properties of the product. Different plant varieties, sugar concentrations, fermentation time, and composition of tea fungus may account for differences in composition and therefore the biological activities would also be affected (Wolfe & Dutton, 2015).

Substrate

Usually Kombucha beverage is obtained from the fermentation of sweetened green or black teas, but some authors (Battikh, Bakhrouf, & Ammar, 2012; Watawana et al., 2016) have studied other substrates as an alternative for its production obtaining interesting results. Battikh et al. (2012) tested the antimicrobial activity of several Kombucha tea analogues finding improved inhibition values than with the traditional beverage, mostly against *Candida* species. Velićanski, Cvetković, and Markov (2013) demonstrated that sweetened Echinacea (*Echinacea purpurea L.*) and winter savory (*Satureja montana L.*) can be used as alternative nitrogen sources, reducing the fermentation time and obtaining comparable characteristics to the traditional beverage. Watawana et al. (2015) fermented coconut water (*Cocos nucifera var. aurantiaca*) with the Kombucha consortium and observed an enhancement of some interesting biological activities. And recently, Ayed et al. (2016) developed a Kombucha beverage from grape juice with improved sensorial and functional properties after just 6 days of fermentation. According to these studies, it can be concluded that investigating the therapeutic potential of Kombucha beverages prepared from different substrates would be an interesting approach.

Time effect

Kombucha tea fermentation normally ranges from 7 to 60 days and the biological activities may increase during this process; however, the best results have been obtained in an average of 15 days (Chu & Chen, 2006). Although most of the antioxidant activities that have been obtained have increased with the incubation time, prolonged fermentation is not recommended due to the accumulation of organic acids, which could reach damaging levels for direct consumption. Furthermore, the CO_2 generated can start to be accumulated at the interface between the biofilm and the broth and may block the transfer of nutrients creating a starved environment (Chu & Chen, 2006). Selecting the duration of the fermentation period also depends on the expected sensory attributes. Reiss (1994) reported that within 6 to 10 days of fermentation a fruit-like refreshing beverage was obtained, contrary to the vinegar taste that is obtained with a prolonged period. According to the Food and Drug Administration Model Food Code for Kombucha brewing (Nummer, 2013) no more of 10 days of fermentation are recommended if produced for human consumption. Coton et al. (2017) studied the evolution of the microbial populations of Kombucha tea from industrial production throughout time (0, 2, 4, and 8 days). They observed that most of the AAB where more abundant in the biofilms than in the liquid broth at day 0 and that after 8 days they arrived to an equilibrium, compared to yeast species that seemed to be quite stable in both phases during the whole fermentation. Chakravorty et al. (2016) evaluated the polyphenol content and the antioxidant activity of Kombucha tea during the course of its fermentation (0, 7, 14, and 21 days) and observed a high tendency to increase specially after the 7th days, which may be due to the higher microbial diversity achieved by that time.

Temperature effect

Maintaining the optimum temperature throughout the fermentation results in a better microbial growth and enzyme activity, therefore, the fermentation benefits are improved. In addition, the antioxidant activity of foods with plant origins can be influenced by temperature variations, for example, the production of phenolic compounds (Hur et al., 2014). Generally, the temperature values of Kombucha fermentation ranges between 22 °C and 30 °C. However, Vitas et al. (2013) carried out the fermentation of milk products with the tea fungus at temperature values of: 37 °C, 40 °C, and 43 °C using optimization models, according to their results the temperature was the most significant factor for the duration of fermentation, and the highest values of antioxidant activity were obtained with temperature values between 37 °C and 42 °C. According to Lončar et al. (2006), quantities of generated acids and metabolites, as well as vitamin C, were greater in samples obtained at higher temperatures.

pH

The pH is one of the most important environmental parameters affecting the fermentation of Kombucha, because some of the acids formed as acetic and gluconic, could be responsible of the biological activities of the resulting beverages. It is also closely related to the microbial growth and the structural changes of the phytochemical compounds which may influence the antioxidant activity (Hur et al., 2014). However, the lowest acceptable pH value should not decrease below 3, which is the one of digestive tract (Lončar et al., 2006). Also, in accordance with Šaponjac and Vulić (2014), to obtain a pleasant sour beverage, the fermentation should be ended when the total acidity reaches the optimum value of 4 to 5 g/L. However, the period of time to obtain this value may differ depending on the origin of the culture medium and fermentation conditions.

Oxygen transfer rate and scaling-up process

Most fermentation processes are aerobic and, therefore, require the provision of oxygen. If the stoichiometry of respiration is considered, then the oxidation of glucose may be represented as:

$$C_6H_{12}O_6 + 6O_2 = 6H_2O + 6CO_2$$

where 192 g of oxygen are required for the complete oxidation of 180 g of glucose. However, both components must be in solution before they are available to a microorganism and oxygen is approximately 6000 times less soluble in water than is glucose, thus, it is not possible to provide a microbial culture with the necessary amount of oxygen for completing the oxidation of the glucose or any other carbon source, in one addition.

At the beginning of the process, significant amounts of ethanol and monosaccharides required for AAB are provided by Kombucha yeasts. The oxidation of ethanol into acetic acid requires one mole of oxygen (32 g) to completely oxidize 1 mole of ethanol (46 g), therefore, the activity of AAB as strict aerobic organisms depends on the transfer of oxygen from the air into the fermentation broth. For that reason, a microbial culture must be supplied with oxygen during growth at a sufficient rate to satisfy the organisms demand (Stanbury et al., 2013). As being a beverage in constant study and evolution, Kombucha has mainly been studied at lab-scale, from 200 mL to 2 L. However, few researchers have studied its fermentation in higher volumes. Malbaša et al. (2006) applied a regression analysis method to a batch process of 8 L and concluded that the pH is a variable that can allow scale-up estimation. Later, Cvetković, Markov, Djurić, Savić, and Velićanski (2008) studied the impact of the specific interfacial area as a variable that could control the production of Kombucha tea, using reactors of 90 L and concluded that reactors having the same interfacial area, although being different in size could provide similar mass transfer conditions. And recently, Coton et al. (2017) worked with volumes of 1,000 L and studied the microbial ecology of the produced tea by meta-barcoding and culture-based methods. They observed that the microbial population seemed not to be affected by the industrial-scale microbial stress factors, which could lead to the standardization of Kombucha tea for industrial production. Besides the volume, there are several parameters for bioprocess development to be taken into account, where the most important include the vessels geometry and the agitation type (Junker, 2004). In Kombucha fermentation, according to some authors, the agitation processing affects the structure of the biofilm due to the reported loss of mechanical strength (Chawla et al., 2009). In static cultures, substrates have to be entirely transported by diffusion and the oxygen availability might become the limiting factor for cell metabolism, which could have a negative effect on the production and quality of cellulose. The kinetic factor that expresses the relationship between the dissolved oxygen and the surface/volume of the medium is the specific interfacial area, which is directly related to other factors, such as the reactor cross-section and the mass transfer coefficient (Cvetković et al., 2008). This means that the rate of Kombucha batch fermentation without agitation and without introducing gas depends on the specific interfacial area. Cvetković et al. (2008) developed a mathematical model to scale Kombucha tea fermentation based on several specific interface areas. The verification of the model was made in reactors of

Table 3–*In vitro* biological activities of Kombucha tea.

Biological assays	Conditions of the tested Kombucha	Results	Authors
Antimicrobial activity	14 fermentation days	Growth inhibition of: *Shigellasonnei, Escherichia coli, Salmonella enteritidis* and *Salmonella typhimurium*	(Sreeramulu, Zhu, & Knol, 2000)
	9 fermentation days	*Helicobacter pylori, Escherichia coli, Staphylococcus aureus* and *Agrobacterium tumefaciens*	(Greenwalt and others 1998)
	21 fermentation days	*Candida glabrata, Candida tropicalis, Candida sake, Candida dubliniensis and Candida albicans*	(Battikh et al., 2012)
Antioxidant activity (DPPH)	10 fermentation days with a mixed culture of acetic bacteria and *Saccharomyces cerevisiae*	60% of inhibition against the radical DPPH with 0.1mL of Kombucha tea	(Malbaša et al., 2011)
	7 fermentation days of Kombucha from Rooibos tea	EC_{50} of 20 mg/kg	(Hoon and others 2014)
	21 fermentation days	IC_{50} of 0.92 mg/mL	(Chakravorty et al., 2016)
Anti-inflammatory activity	8 fermentation days of Oak infusion	Suppression of the proinflammatory cytokines TNF-alpha and IL-6	(Vázquez-Cabral et al., 2017)
Anticarcinogenic potential	14 fermentation days with subsequent solvent fragmentation (chloroform, ethyl acetate and butanol)	Cytotoxic effect of 21.5% in 786-O and 93.45% of inhibition of U2OS cells with 100 μg/mL, reduced cell motility in A549	(Jayabalan et al., 2011)

large volume (90 L) and very small vessels of 0.33 L. The model standardizes the optimal conditions as: 70 g/L of initial substrate (sucrose), interfacial area of 0.0231 to 0.0642 cm^{-1}, and 14 days of fermentation. They concluded that regardless of the vessel size or volume, if the value of the interfacial area is constant they could ensure the production of Kombucha tea with similar properties. In the specific case of batch fermentation of Kombucha tea, several biological factors should be taken into account. Especially in the absence of agitation, where a microbial disintegration may occur between the aerobic acetic bacteria which will tend to occupy the surface layer and the yeast which may precipitate to the bottom of the vessel (Lončar et al., 2006), and this might have negative effects in the fermentation process. Besides the fact that microbial cellulose has been already well studied by some authors (Campano et al., 2016; Czaja et al., 2006), the available information defining the optimal reactor conditions for its development such as surface/volume or surface/height are limited. In order to investigate the influence of the volume in the processing, Lončar et al. (2006) worked with several conditions and found that the best geometric conditions for scaling-up the fermentation were obtained with a reactor of 4 L and a diameter of 17 cm. Goh and others (2012) investigated the relationship between the yield, the properties of the biofilm produced from Kombucha fermentation, and the surface area, and found that the production of the biofilm was increased with an intensification of surface area, and was decreased with a broader depth. This can be explained because the metabolic process is completely aerobic and it is constantly generating carbon dioxide, which might be trapped in the pellicle and end up being accumulated in greater quantities especially in the deeper mediums. However, Caicedo, Da França, and Lopez (2001) found that even though the surface area is determinant, the height is not unimportant, since it was observed that a minimal height is needed for the development of the pellicle, this taking into account the production of several layers of cellulose throughout the fermentation which will occupy part of the initial volume.

Beside all the previously mentioned parameters, Kombucha's elaboration process may also affect its final properties. The process still remains quite artisanal and the exact proportion of components may vary depending on the expected product. However, it generally follows the next order: Tea leaves or plant extracts are added to boiling water and allowed to infuse for about 10 to 15 min after which the leaves are removed. Sucrose is next dissolved in the hot tea and after the infusion is left to cool. The sweetened beverage is subsequently poured into a container and inoculated with about 3% w/v of already prepared Kombucha biofilm, afterwards the container is covered with a clean cloth and incubated at room temperature.

Nevertheless, in order to optimize the industrial production of Kombucha tea, as being a functional beverage, a complete study including high-volume production, microbiological identification, and biological assays should be performed.

Biological Activities

Tea is consumed in China since 5,000 years ago, it is the second most popular beverage after water, and it is even considered as the oldest known medicine because of its health benefits. Torino et al. (2013) reported that the bioconversion from conjugated forms of phenolic compounds into their free form during fermentation improves their healthy function. Several investigations have been done in order to understand the impact of tea fermentation with the microbial consortia and some of its biological activities have seen to be improved (Table 3).

Even though most of the biological assays in Kombucha tea have been done *in vitro*, several authors have done *in vivo* studies using rats and have found interesting results. Srihari, Karthikesan, Ashokkumar, and Satyanarayana (2013) evaluated the antihyperglycaemic efficacy of the fermented tea in diabetic rats by testing different concentrations of Kombucha extracts during 45 days. They observed that with the daily administration of 6 mg/kg bw the glycosylated hemoglobin was decreased and the plasma insulin was increased. Bhattacharya, Gachhui, and Sil (2013) studied the protective effect of Kombucha in different organs including pancreas, liver, kidney, and heart of diabetic rat models and the obtained results showed significant antidiabetic potential which allowed the restoration of the induced pathophysiological changes. Later, Gharib (2014) subjected a group of rats to electromagnetic waves increasing the iron copper levels and decreasing the zinc content. The rats were then administered with Kombucha tea during 9 weeks and a decrease in the iron content was observed, going from 65 to 99.5 μg/g. This allows to conclude that Kombucha could have ameliorative signs against the effects of electromagnetic radiation. And recently, Bellassoued et al. (2015) worked with rats fed with cholesterol-rich diets and high thiobarbituric acid reactive substances (TBARS) levels, and found that the TBARS

concentration was significantly reduced in the liver and kidney after the treatment with the fermented tea. Their results showed that Kombucha tea could be considered as a therapeutic agent against liver and kidney toxicities. Although several *in vitro* biological activities have been studied for Kombucha tea, still clinical investigations and more *in vivo* evaluations should be performed in order to confirm the claimed health benefits of the beverage.

Potential Toxicity

Kombucha fermentation is commonly homemade, and therefore it is important to be cautious because pathogenic microorganisms can contaminate the tea throughout the preparation. Some cases of health disorders have been reported by some individuals with suspected dizziness and nausea, severe illness, allergic reactions, and head pain, thus leading to its contraindication in pregnant and lactating women (Jayabalan et al., 2014; Srinivasan, Smolinske, & Greenbaum, 1997; Watawana et al., 2015). On the other hand, Vijayaraghavan et al. (2000) evaluated oral toxicity for 90 days in rats and any toxic signs were detected. The U.S. Food and Drug Administration also carried out some tests and reported that Kombucha tea is safe for human consumption. However, because of the previously mentioned reasons and to the microbiological complexity of this beverage it is always important to produce it under the FDA Model Food Code (Nummer, 2013).

Conclusion and Perspectives

Even though nowadays Kombucha tea is known all over the world, its biological properties are not well understood. More research concerning Kombucha fermentation kinetics is needed in order to be able to identify the produced metabolites, especially those that may be potentially beneficial and to understand its relationship with the biological activities. Regarding the Kombucha substrates, plant extracts have attracted great interest because of their multiple applications. Furthermore, the extension of a fermentation process from a laboratory scale to a commercial product is a challenge because of the difficulty of evaluating the factors which may influence the scaling process during cultivation. More scientific research should be done to understand the links between the fermentation and the biological activities of Kombucha tea, establishing it as a functional beverage with a clear evidence in the advantages and disadvantages of its consumption. There are a number of parameters and variations to be measured, controlled, and experienced in order to determine the optimum fermentation conditions.

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Author Contributions

Silvia Alejandra Villarreal-Soto wrote the manuscript under the supervision of other authors. Patricia Taillandier, Sandra Beaufort, Jalloul Bouajila and Jean-Pierre Souchard refined and reviewed the draft for publication.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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