BDAS, LLC WPSP#2 - Oct. 2015

So What is Kombucha? An Alcoholic or a Non-Alcoholic Beverage? A Brief Selected Literature Review and Personal Reflection.

Gary Spedding, Ph.D.

[Brewing and Distilling Analytical Services, LLC, 1141 Red Mile Road, Suite 202, Lexington, KY 40504.] Tel. (859) 278-2533. Website: www.alcbevtesting.com.
Correspondence: gspedding@alcbevtesting.com

Abstract-Overview

Kombucha is a complex beverage touted to be both refreshing and health-promoting; almost ascribed to the status of a cult-like "cure all". Yet, despite its long history (and incredible popularity as a home-made beverage - especially over the last 50-100 years) it remains largely a mystery (at least outside the academic literati) as to how all the microorganisms inter-play to create a diverse metabolic soup and highly acidic (sometimes throat-burningly so) beverage. Indeed only within the last two years have the majority of microorganisms involved in the gelatinous mass (a biofilm known in the business as a Scoby – "symbiotic community of bacteria and yeast" or zoogleal mat) been finally identified through modern molecular genetics. Furthermore, while a few papers have touched upon the metabolic properties, - the chemicals present in Kombucha and Kombucha-like beverages for example, - there is a solid lack of understanding as to: the true concentration of key components; to the best practices for the sensory evaluation of these products; and to the potential for both alcohol and acetic acid production (the dominant acidic note) in stated beverages.

Even qualified microbiologists sometimes have difficulty in maintaining pure cultures of organisms and have done little metabolic follow-up research with commensal populations in alcoholic beverage production (except perhaps in the Belgian brewing industry and for certain other sour and spontaneously fermented brews – from which valuable lessons may be learned? See for example ref. 15). And yet home-kombucha producers, with no knowledge of microbiology or sterile technique are producing an unregulated food-product, often under less than pristine food-quality manufacturing practices. Moreover, they are not aware as to what is happening with fermentation nor how to control it – to the point that they believe they are creating a non-alcoholic and safe-to consume potable beverage. In dealing with multiple organisms, working in a highly intricate ecological commensal fashion, the maintenance and propagation of a uniformly (microbial) populated scoby is likely next to impossible outside a clean – working environment. This then makes for difficulties in producing a consistent quality product and with inherent dangers if the scoby or sweetened tea used for fermentation becomes contaminated with other naturally airborne microorganisms. Microbial food spoilage itself forming the subject of several lengthy volumes in the literature.

Now Kombucha societies are, quite alarmingly, under the naïve impression that these beverages are so complex in composition that standard official techniques for measuring alcohol are not in fact determining true levels – assuming, in unsubstantiated ways, that the readings are falsely high. In fact this author firmly believes that many Kombucha producers are not even aware that alcohol is being produced at all or, if they are cognizant of this aspect of metabolism, that they believe *de facto* that all of it is converted into acid (largely acetic acid). The author here argues that if any false readings are involved they would more likely in fact be erroneously low with certain components possibly "masking" alcohol when measured using certain methods (based on personal experiences in measuring complex distilled beverages and highly acidified alcohol mixtures). However, our research indicates that neither artificially low nor high readings are common

in practice. Most methods do in fact measure the true alcohol content of these beverages.

A full education as to what constitutes these beverages and an understanding of the ecology, microbiology, metabolic biochemistry and chemical kinetics – along with the analytical chemistry to evaluate them - is needed to better define and understand these type beverages. While it is possible, with a very careful approach and a full understanding of process, to obtain non-alcoholic kombucha-like products (below 0.5% alcohol by volume) many products will (and do) in-fact contain significant levels of ethanol. Producers and the consuming public need to be aware of this fact. The product if made for commercial purposes needs to be periodically tested by appropriate regulatory agencies to maintain compliance. A lack of rigorous testing of commercial scale products is a responsible factor for many kombucha brands being consumed with alcohol levels far above the 0.5% alcohol by volume (ABV) limit as classifying them as non-alcoholic beverages. US regulatory authorities recalled all such products a few years ago from the market pending evaluation. Unfortunately many have crept back on the market and have clearly not been tested for actual ethanol content (or continued fermentation in bottle leads to higher ethanol content over their typical or extended shelf-life). Moreover, for as yet unknown (to the author) reasons, much of the claimed nutritional information on product labels is also not correct. If products do contain more than 0.5% ABV and the true ABWt (weight) is not determined then the calorie values will never be reported accurately enough for regulatory compliance. Calories are based on sugars remaining, and protein and alcohol by weight for official purposes but are also in fact dependent upon any fatty (acid) materials and organic acids which are (obviously by definition) high in kombucha-type beverages. [Further details on nutritional content available via consultation with the author – no additional commentary being provided here.]

The following discussion provides a brief, personal and directed review of most of the significant scientific literature on the topic (most from the last two decades). Where repetition could be prevented, in citing earlier article-related facts and statements the most up-to-date articles, also cross-referencing the earlier works, are chosen to represent the points to be made; a fuller set of reference titles is available to those interested and who do not have ready access to the scientific journals. It is hoped that this article and the selected references will clarify the issue with respect to some myths and misconceptions and point out the deep need for further research into kombucha production and the regulatory control of the manufacture of such food/beverage products. This author's own bias is towards an understanding of the alcohol content of such products and, as will be seen, studies are lacking in this area. The microbiological side of the story is only now becoming better

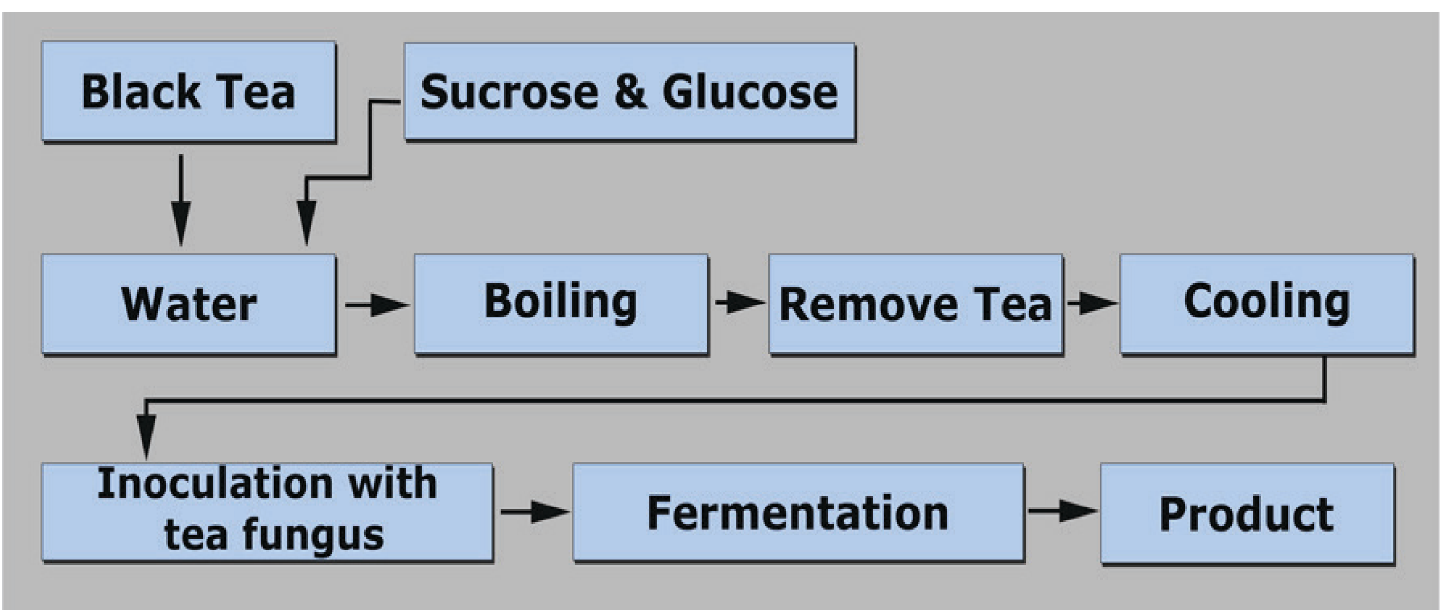

Figure 1. An overview schematic of kombucha production. Adapted from 16.

understood as are the details regarding the potential health benefits ascribed to many metabolites produced by the "tea-fungus" – but, unfortunately, not the key metabolic aspects of alcohol production. This latter topic is suspiciously lacking in the research field or especially lost in all the rah-rah support of these "cure-all ailments" beverages.

1.0. A literature Review on Kombucha

Kombucha has been discussed both in the popular (free-press) literature (O'Niell, 1994, ref. 1) and in many scientific publications (see full references cited below and in the final references section) including several key medical journals. Yet that peer-reviewed literature is scattered widely and not readily available to the millions of people making this beverage either as home-brewers or as commercial producers (1). Consequently a lot of myths and misconceptions abound about this most complex product. Two key reviews are recommended discussing all facets of this huge topic and reflecting many of the points mentioned herein. An earlier review by Dufresne and Farnworth in 2000 (2) and a much more recent one by Jayabalan, *et al*, in 2014 (3) are recommended as starters and for those who do not wish to get bogged-down with the scientific technicalities and experimental details in the multitude of scientific articles out there. Another earlier review-type article, mainly dealing with the microbiology of the scoby or tea fungus but also considering claimed health effects, is that by Greenwalt, *et al*. from 2000 (9). These reviews cover the basic history of Kombucha, the production of the beverage along with the summarized science, health benefits and potential health hazards of Kombucha consumption.

1.1. Kombucha Briefly Defined

- *"Kombucha is a sweetened tea beverage that, as a consequence of fermentation, contains ethanol, carbon dioxide, a high concentration of acid (gluconic, acetic and lactic acids) as well as a number of other metabolites and is thought to contain a number of health-promoting components." (4).*
- *"Ultimately, it would appear that the naturally low pH and ethanol content of the beverage generated under regular, household brewing conditions, combined with other forms of competition involving the indigenous microbial population, is sufficient to limit contamination from undesirable populations." (4).*
- *"Kombucha is one of a number of tea-based beverages presumed of Asian origin fermented by a mixed culture of bacteria and yeasts, together forming a surface mat or pellicle which is known as the "tea fungus". Besides kombucha it goes by many other names often with "cha" in the name (with "cha" related to the tea component of stated beverages)." (11)*

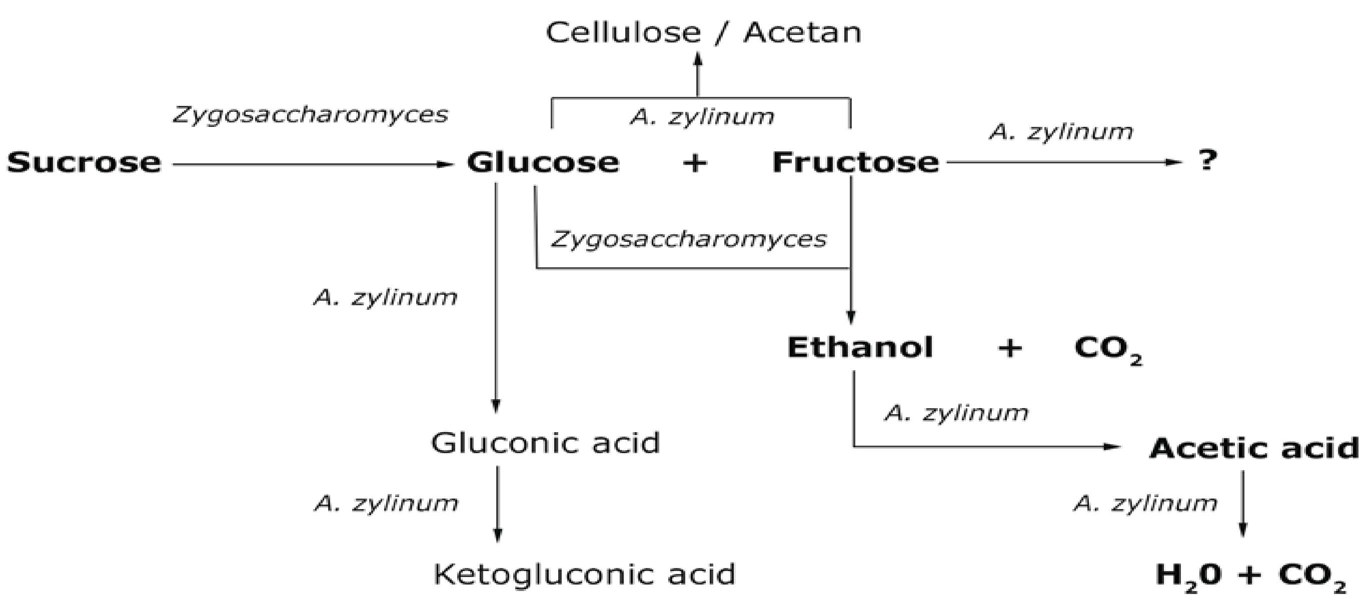

Figure 2. A basic scheme of important metabolic activities of the *Acetobacter* (bacterial strains) and the *Zygosaccharomyces* (yeast) species isolated from a tea-fungus. Adapted from Sievers, *et al,* 1995 (5). Yeast hydrolyze sucrose to glucose and fructose by the enzyme invertase and produce ethanol via glycolysis, with a preference for fructose as substrate.

• *"Biofilm, commensal mat or biomass, tea-fungus, scoby and zooglea are just a few names for the complex group of organisms and cellulose matrix that holds them together in a rubbery disk-like object or floating strands of matter."*

O'Niell in 1994 (1) provided one of the first popular press descriptions of this beverage: "Kombucha is brewed by placing the fungi in a glass bowl with three quarts of cooled black tea that contain one cup of sugar. It is then covered with cloth and placed in a dark, warm place for a week to 10 days, until it spawns a second disk. The tea is then strained and stored in glass in the refrigerator" (1). This is likely an oft quoted and used recipe for home Kombucha producers. Green tea can be used. A web search reveals a half dozen or so popular press books perhaps giving a little more precision to recipes and production (the author has not seen or reviewed these type publications). A few more scientifically defined formulas appear in the literature but are likely not viewed by many kombucha producers. The apparent simplicity of production leading to a lack of understanding of the complexity of these beverages. The reader just interested in the general facts and myths and an earlier discussion on the US craze for Kombucha will find enough in the New York Times article by O'Niell (1). Though do consider the three reviews noted above (2, 3 and 9). Figure 1 shows a brief schematic dealing with Kombucha production and has been adapted from Mo, *et al*. (2008) (16).

According to O'Neill (1) the craze for Kombucha in the US began in 1992 with an article in publication called Search for Health. The author of that article, T. Valente, also started to distribute perhaps the first popular press book on the topic. "Kombucha: Healthy Beverage and Natural Remedy From the Far East" by G. W. Frank, 1991 – published in Austria (cited in 1).

Many on-line sites and popular books are available but for those serious enough about producing such intricate beverages a reading of some of the literature described below should be considered essential reading.

In addition to producing and providing many potentially beneficial components; mineral ions, amino acids, organic acids, anti-oxidants and vitamins, kombucha fermentations are started using a "fungal mat" (a complex mass of fungi/yeast and bacteria held together in a complex cellulose network). The culture has gone by many names including the tea-fungus, teakwass, a zoogleal mass or mat, the magic mushroom or the scoby to name but a few (1, 3 and 10). This mass is reproduced with each successful round of fermentation to produce, in popular parlance, the babies (1) – these are sold or passed around to other producers but whether they can be maintained consistently remains doubtful at best. What has been found is that the populations of organisms residing within the tea-fungus cultures varies from country to country and probably from region to region (see for example 15 and 18). This makes dangerous interpretations by new (untrained) producers in thinking that all their fermentations will be similar if following standard recipes for the teas and the sugar (usually sucrose) used. The use of fruit, herbs and vegetables adds further complexity into the mix and may affect the metabolic flux considerably (little research is available covering this aspect of the topic yet many fruit and other-flavored kombuchas flood the market every year).

Two of the first reviews concerning Kombucha and health in the scientific literature are those by Dufresne and Farnworth (2000) (2) and by Greenwalt, *et al*. (2000) (9). Described as "a refreshing beverage obtained by the fermentation of sugared tea with a symbiotic culture of acetic bacteria and fungi, consumed for its beneficial effects on human health." (2). In their review Dufresne and Farnworth briefly review early research conducted in Russia over 100 years ago and cite a number of its ascribed health benefits. Citing also Kombuchas' possible origins in China (noting the date 220 BC as of significance in its acceptance) and its spread to Korea, Japan and Russia and then to Europe (2). A little more on the history is presented by Greenwalt, *et al.* (9) and in the 2014 review by Jayabalan, *et al*. (3)

Providing tea chemistry details and a more well defined use of sugar (50 grams/L) Dufresne and Farnworth (2 and the optimal 50 grams/L substrate use cited also in 3, 6 and 9) also state that the steeped tea is acidified with the addition of vinegar or a portion of previously prepared Kombucha prior to the addition of the "tea fungus". The conditions for fermentation are prescribed and they state that the taste of Kombucha "changes during fermentation from a pleasant fruit-sour like lightly sparkling flavor after a few days to a mild vinegar-like taste with prolonged incubation" (2). The prior partial acidification of the tea might be news to many Kombucha folks and might, like for Bourbon production, provide a controlled environment to prevent unwanted microorganism activity in the tea? However, this detail perhaps underscores the need for careful management and consistency in manufacture.

1.2 Health Claims and Counterclaims

This review is not intended to focus on the health-promoting claims touted for this type beverage. However, a few references are available for the interested reader to consult. Cogent discussions are provided for example in the reviews by Dufresne and Farnworth and Jayabalan, *et al*, (2 and 3). This includes the necessary bioavailability issues with respect to nutrients, probiotics and minerals and vitamins.

A few cases of health disorders have been associated with kombucha and it is recommended that copious amounts of water be also consumed with kombucha based on potential toxic reactions and ingestion of the huge amounts of acid present in these beverages (2); so much for kombucha alone be a thirst quenching beverage? A reading of the three key reviews mentioned above (2, 3 and 9) and perhaps consultation with physicians would be in order regarding the touted health benefits of consuming such highly acidic and possibly alcoholic (see below for more on this) beverages? Certainly (after reviewing several medical journal articles or as cited in the reviews) indiscriminate consumption seems generally undesirable for certain groups and requires careful consideration of advantages and disadvantages associated with such consumption.

1.3. Kombucha Composition and Basic Chemistry and Biochemistry

In simple terms kombucha fermentation entails the transformation of sucrose into glucose, fructose, gluconic acid, ethanol, and acetic acid (5). Glycerol is also noted as being produced (2) and lactic acid (6). Fermentation of the sweetened tea (black or green tea) is initiated by the addition of a portion of a previous culture – the complex tea-fungus (aka, zoogleal mat, scoby). The pH of the culture broth falls from 3.75 to 2.42 as a result of the production of both acetic and glucuronic acids (5). The actual metabolite composition and concentration depends on the tea fungus source, the sugar concentration, and the time course of fermentation (2). 50g/L sucrose has been stated to be optimal for both ethanol and lactic acid production (2, 6 and 9).

Figure 2 provides details of sucrose utilization, and ethanol and acetic acid production. The scheme is, in this author's opinion, often mistakenly interpreted by non-scientists as indicating a complete conversion of the generated ethanol into acetic acid. Less ethanol might remain after complete sugar utilization if the acetic acid is further converted to water and carbon dioxide. However, the generation of more carbon dioxide, once the kombucha is bottled, is also a cause for concern in over-pressurizing the bottles. Studies are lacking on the amount of alcohol produced and present in bottled and aged bottled products (see section 1.5). A fermentation balance of substrates sucrose, glucose, fructose to products ethanol, acetic, gluconic acid and CO_2 was determined by Sievers, *et al*. (5) and is discussed a little further in Section 1.5 with respect to ethanol evaluation.

Yeast and bacteria in the tea fungus make use of substrates by different and complementary ways. Yeast cells hydrolyze sucrose into glucose and fructose, and produce ethanol, with a preference for fructose as a substrate (5, see Figure 2) (An interesting question then is raised here. Do Kombucha producers ever use high fructose corn syrup for fermentation? That speculatively could tip the balance in favor of alcohol production? To the best of knowledge by this author there is a lack of testing of such issues though different substrates for fermentation were described by Reiss (6) and by Malbasa *et al*. (7 and 8); see also under Section 1.5. Acetic bacteria utilize glucose to produce gluconic acid and ethanol to produce acetic acid. Lactic acid synthesis is proposed due to the action of lactic bacteria on ethanol and acetic acid (2). "More complex interactions probably occur but have not been elucidated." (2). Indeed these tea-fungus commensal systems, and the conditions applied and formulas for kombucha production are incredibly complex. With multiple organisms and substrates involved in the production of acids and alcohol alone complex modeling or investigations of fermentation activity is needed. In reviewing the literature it is clear that there is more interest in other properties of kombucha (and other biological activities, 2, 3 and 9) and only a handful of reviews actually discussing alcohol production in any seriousness.

With the complex population of organisms it is by no means assured that all the sugar gets converted into acetic acid (via ethanol) as seems to be assumed by the lay-person kombucha "expert". In the author's opinion much more fermentation chemistry and analysis needs to be undertaken especially now that the major core organisms have been identified (see section 1.4 on Microbiology). Acetic acid stimulates the yeast to produce ethanol and ethanol in turn can be helpful to acetic acid bacteria to grow and produce acetic acid (cited in 2). Both ethanol and acetic acid are reported to exert antimicrobial activity against potential pathogenic bacteria, thus helping protect the tea-fungus from contamination (2). Despite interesting statements like these which catch attention from the health perspective but do suggest that alcohol is a key player in preventing unwanted antimicrobial contamination the details of the metabolic interplay and alcohol production and consumption in the kombucha ecosystem has seemingly been missed in most of the research to date.

1.4 Microbiology

The microbiological composition of the tea fungus has been investigated – with early work summarized (2) – more classical microbiological detection methods detailed (9-16) and with yeast also more specifically covered in detail (15 and 17). The early classical microbiological approaches are now complemented by more rigorous analysis as presented only within the past two years (4 and 18). Bacteria and the "fungus" (fungi and yeasts) present in Kombucha are said to form a powerful symbiosis able to inhibit the growth of potential contaminating bacteria (2 and references cited therein). Acetic acid bacteria (*Acetobacter* and "glucono species" *Gluconacetobacter*), *Lactobacillus* (lactic acid bacteria or LAB) and yeasts including *Schizosaccharomyces, Saccharomyces, Kloeckera/Hanseniaspora, Torulaspora, Zygosaccharomyces, Brettanomyces/Dekkera, Candida* and *Pichia* species are known to be present (2-3, 15, 17 and 18). The detection of a number of *Lactobacillus* species being a surprisingly recent finding (4, 18) as was the finding of previously unreported yeast species (4). Of possible significance here is the different population characteristics of Kombucha tea fungus obtained from different countries (4 and 18)! Different populations might produce different acidity levels (lactic as well as acetic acid) and thus potentially impact the final ethanol levels?

Now that the level of information has risen with respect to microbial populations, the note above about the time to follow up with more in-depth studies of fermentation and to fundamental metabolite analyses in these different tea fungus products is reiterated. This may well bear on non-alcoholic and fully alcoholic examples of Kombucha as found on the market. It might not be a simple matter to adjust final alcohol levels by simply varying the sugar content in a fermented tea recipe for example (see Section 1.5). The selection of tea-fungus cultures with a greater proportion of less rather than more fermentative yeasts might well be of benefit (or at least interest) to non-alcohol Kombucha producers. "Investigation of fermentations produced by defined starters is required to elucidate the exact contribution of each yeast to the final flavor and biochemical composition of kombucha." (4). One size does not fit all! Also of interest here being the work of Marsh and coworkers which showed the possibility of more troubling bacteria present, possibly via contamination, in several commercially obtained samples (4).

Many lay folks think the complex mass of organisms that usually forms a pliable-rubbery-like mass – in the form of a disc (a biofilm known in the industry as a Scoby) is a mushroom (1). As noted above it is in fact a commensal population of yeasts and several bacterial strains living in a truly symbiotic community (1). As such the metabolic interplay and the time sequence of activity of the many organisms form an as yet poorly understood ecology (though see 15). This means that the control of production of kombucha is paramount for consistency. Do all the organisms act in the same manner every time? Are metabolic components uniformly produced by each respective organism and uniformly consumed by others in each fermentation? Are fermentation conditions (nutrients and sugars – sucrose is largely used for the fermentation) the same each time? How is this composition monitored or how should progress of fermentation be monitored? Temperature (and control), duration, volume of production and even seasonality (natural microbial populations in the air as potential contaminants of the scoby culture) and sanitation are all factors to be taken into account. The product is a "live culture mix" and activity does not cease upon bottling and, furthermore, the product is never pasteurized (nor is this a desirable process for Kombucha – a topic not dealt with here but covered a little in the literature). Residual nutrients (sugars, the minerals and vitamins touted to be in the product, and oxygen content etc.,) will all play a role in continued fermentation and metabolic activity in bottled product (also discussed a little further in Section 1.5). This continued activity includes equilibrium exchanges between acetic acid and ethanol, possible further production of acids and ethanol and other components and carbon dioxide gas production. Manufacturers of this product often state "keep refrigerated" – never allow to warm up and "never shake the product". This is to prevent further gas pressure build up and potential rupture of the glass packaged product caused by violent gas release – enhanced by the particulate matter in solution forming nucleation sites (centers of accumulation) for gas to form and build up. This release is what causes fobbing or gushing of beverages and product loss for consumers when not carefully controlled for. In addition to alcohol content this potential for rupture or explosion of bottles needs to be more carefully regulated. Perhaps pressure-release-type caps should be enforced for such mixed live-culture beverages in addition to keeping product chilled?

Fully qualified microbiologists are only now uncovering all the complexities of this symbiotic culture. The scoby varies in microbial composition and the full complexities and progression of activities of each organism are still not understood. How each affects the other especially with regard to all the complex metabolic pathways involved may never be fully resolved. However, commercial examples should be subject to further scrutiny and conditions established to monitor the consistency of beverage production with respect to several metabolites that may affect the consumer. "A better understanding of the ecology of fermentation will enable the development of combinations of yeast and bacterial strains to provide a product of predictable taste and consistent quality." (15). Understanding the control of metabolism in simple systems is becoming better known – not so much yet in complex systems (26). Yet for kombucha production many species of bacteria and fermentative yeasts play a role. As will be seen acid production has been studied in isolation from ethanol production. The acids produced are easily tested for by lay-scientists and kombucha producers but alcohol is not. Unfortunately ethanol studies are limited. That is the subject of section 1.5.

1.5 The (Limited) Alcohol Studies

To say that relatively few studies have been undertaken to seriously address the alcohol content issue in kombucha-style beverages is an understatement. The few studies (some detailed below) that have been undertaken simply measured the alcohol content as a part of a larger survey of metabolite investigation and then only on a laboratory scale. More significantly these were done under highly controlled conditions. Production scale operations by inexperienced personnel may lead to product with unknown alcohol content. In fact many kombucha producers are rather surprised when told they are well above the 0.5% ABV (alcohol by volume) level and that their beverages cannot be classed as non-alcoholic. As will be seen below a few laboratory studies do show that alcohol produced during fermentation will fall later in the process to less than 0.5% ABV (for traditional formula kombucha-type fermentations!). However, no one has followed continued fermentation while product sits on store shelves. One sad fact is the total lack of discussion on alcohol by the authors in a recent comprehensive (and more accessible) review of kombucha (3). No wonder many do not think about alcohol as a factor in such beverages?

However, a paper presented around the time of the initial growth in popularity of kombucha in the US may provide clues as to why the generally held view is that these beverages are non-alcoholic in nature. The authors Srinivasan and Smolinske, (19) cited a paper by a P. Staments from a 1994 paper in "Mushroom-The Journal" as evidence for the 0.5% ABV value. This author has not yet seen a copy of that work though it will be important to see if that paper represented qualifying statements from other significant works or observations on this topic or is just mere "hear say" (a lot of earlier popular literature in many languages remains out there for viewing if it can be found, translated and interpreted!). How such values for ethanol content were derived with more modern production of kombucha (last 100 years) and the sources of the Staments' comments will clearly be of more than a passing interest.

Notes on those studies that have attempted to look at alcohol levels in kombucha now follow. A reminder from section 1.3 – alcohol is produced from glucose and fructose and is consumed to produce acetic acid. The predominant species *Brettanomyces, Zygosaccharomyces* or *Saccharomyces* are the primary fermentative microorganisms involved (see Section 1.4). In kombucha fermentation the role of yeasts is to invert sucrose and form ethanol, which *Acetobacter* species then convert to acetic acid. Fermentative activity of yeasts may be inhibited by the acetic acid produced so this might actually limit ethanol production - though this might not be until levels of from 6 to 18 grams/L of acetic acid are attained (11). In commercial production the collective population of organisms and fermentation conditions will be very different from the lab-scale trials noted below and as such the data noted should be taken as a guide only and not substitute for a lack of investigation of the ethanol content in commercial production.

Ethanol production of between 0.63 g/100ml and 0.811 g/100 mL (approx. 0.8-1.0% ABV) was noted in studies by Reiss (6). This was measured using an accurate enzymatic method (normally reserved for detecting low levels of alcohol in low to non-alcohol containing foods and drinks) but from a highly controlled culture on a small lab-scale. The greatest significance here is that 50g/L sucrose gave the highest amounts of ethanol and lactic acid and exactly this sugar concentration has, according to the authors, been used in traditional recipes for the production of "teakwass" (another name for kombucha) (6). Interestingly, the use of more or less sucrose gives less alcohol - likely for complex metabolic reasons. Whether shifting to the paradoxically higher amounts of sucrose for expected lesser alcohol production works in actual production scale remains to be seen. Another paper dealing with fermentation balance is that by Sievers, *et al* (5) and should be consulted by those interested in the "alcohol problem". In that work, using 70 grams/L of sucrose for fermentation in a small-scale system, ethanol content reached a maximum of 9.1g/L (0.91 g/100 mL = ca. 1.15% ABV) after 24 days of incubation and then decreased to 0.7 g/L (0.07 g/100 mL = ca. 0.1% ABV) after 62 days. Ethanol was oxidized to acetic acid, with the acid rising from an initial value of 0.9 g/L to 28 g/L after 40 days. At that time it was slowly over-oxidized to water and carbon dioxide (5, see Figure 2). Liu and others used high performance (HPLC) chromatographic analysis to show that kombucha fermented teas contain glycerol, acetic acid and ethanol and suggested that ethanol stimulates *Acetobacter* species to produce acetic acid which, then in their turn, stimulate yeast to produce ethanol (14). Furthermore, they seem to be among the first groups to suggest that the ethanol and acetic acid produced by yeasts and *Acetobacter* might prevent competition from other microorganisms and thus may afford some protective effect against contamination or growth of unwanted organisms (14).

Another study utilizing HPLC to look at ethanol production – that of Blanc (1996) (20) showed that only 0.134% ABWt (alcohol by weight) or approx. 0.17% ABV was produced after 5 days incubation with 100 g/L sucrose (see note above on more sucrose leading to lower ethanol). This was in contrast to the work of Reiss (6) but Blanc did point out that Reiss indicated that different compositions of tea preparations depend greatly on the individual tea fungus used (20). This is a point mentioned in section 1.4 with different kombucha cultures from around the world potentially producing radically different product compositions.

This variability in composition and population of organisms was also discussed by Greenwalt, *et al.* (21) who presented notes on the history and microbial population of kombucha colonies and posited that the tea fungus contains variable microbes as shown via analyses from cultures or "scobies" collected from around the world. They also proposed that acidity levels at around 33g/L total acid limits the ability of many other organisms, including contaminants, to grow. However, the acidity can get too high and pose a potential risk if consumed. So a balance of acidity and ethanol is required.

Greenwalt, *et al*. (21) measured ethanol also by the enzymatic method. In this work, using 100g/L sucrose, kombucha colonies produced 3.3% total acid, 0.7% acetic acid, 4.8% glucose and 0.6% ethanol (presumably by weight by nature of the assay and the other values reported below) after a 9 day fermentation. When fermentation went beyond the desired endpoint acidity reached 24g/L as acetic and with 14g/L ethanol determined. This is 1.4 grams per 100 mL or 1.4% by weight ethanol (not correcting for the sample SG this would be about 1.8% alcohol by volume). An interesting point here is that even at the desired termination of fermentation according to these authors the ABV is slightly above 0.5% ABV. If the fermentation goes too long under these conditions (or perhaps with continued fermentation in bottle) the alcohol is likely to be much higher and potentially to levels we sometimes see in commercial products tested off-the shelf and prior to the noted expiration date. Once again these were laboratory–scale experiments of traditional tea and sucrose ferments – not covering extra nutrients or sugar contents from flavorings, fruits or vegetable matter used in current commercial examples.

In a later paper Greenwalt, *et al.* (9) make the statement that the alcohol content of Kombucha is thought to never exceed 10g/Liter (potentially 1.26% ABV and similar to the 1.8% proposed above). Interestingly for a US Kombucha producer (Laurel Farms of Florida) whose products or cultures were featured in the data set (and also covered by O'Neill, 1) the authors did not present an alcohol value. These commercial (fermented) examples showed similar acetic acid and gluconic acid levels as reported for another test sample which gave about 0.7% ABW during fermentation (again approx. 0.9% alcohol by volume). The data were thus incomplete with respect to alcohol production in a commercial setting as far as this review study was concerned and this author is suspicious as to why such alcohol data were either not determined or reported; as a review all the relevant data of course might not have been available to the reviewers. This article was nevertheless far from complete in its evaluations. Though it must be stated that other authors using sophisticated auto analyzers in studies aimed at looking for the antimicrobial effects of kombucha also failed to detect either lactic acid or ethanol in their studies (23). The question to ask is why not? The methods, as listed in the limited studies, used to determine alcohol are not without their difficulties in testing nor the best or complete set of methods available to measure ethanol; though the enzymatic method is suitable to very low volume lab-scale testing. The topic warrants immediate further and extensive investigation on larger scale cultures.

Another interesting paper that would have enhanced our understanding of acid vs. alcohol production actually and surprisingly failed to look at alcohol as an important metabolite. The study by Jayabalan, *et al.* (24) detailed the changes in content of organic acids and tea polyphenols during kombucha tea fermentation but despite an interesting and detailed study makes no mention of alcohol. Two other studies (refs. 7 and 8) also proved of disappointment to this author. The works by Malbasa, *et al.* (7 and 8) could have provided very nice evaluations of acidity versus alcohol production but they also failed to determine the alcohol levels in their otherwise interesting experiments using different sugar sources for fermentation. Similar experiments might be worthwhile while also addressing relative levels of acetic and lactic acids and ethanol especially in larger scale commercial operations. The overriding conclusion seems to be that while there has been a lot of research into supposed health benefits of kombucha from a compositional viewpoint there is, perhaps suspiciously, a dearth of crucial information on alcohol production and stability in such beverages.

Yet another paper which dealt with scaling up Kombucha operations also failed to look at alcohol production (24). However, it provides great insights into dealing with conditions moving away from pilot scale to large scale operations. Measurements of pH and acidity were made and mathematical models constructed. The observations of the need for oxygen for acetic acid bacteria to perform efficiently should not be overlooked in acid and ethanol production. Significantly these authors noted that, contrary to popular practice among kombucha producers, the pH value cannot be used as a critical parameter to determine the end of sweetened tea fermentations; this role is better played by monitoring total acidity (TA) (24). Other authors (25) also argued that pH values should not be considered as significant for monitoring progress since carbon dioxide produced during the fermentation may perform buffering effects. Also suggesting that total acidity might be a better indicator to terminate the fermentation and prepare for packaging (25).

Quoting from Cvetkovic, *et al*. (2008, 24): "Expanding a fermentation process from the lab-scale unit to a commercial one is a challenge because of the difficulty in assessing the factors that affect the scale-up process during the cultivation." (24). Cvetkovic and team discuss an initial need for significant amounts of ethanol and sugars to feed the acetic acid bacteria and the factors and kinetics needed to drive acid production. If these factors are not optimal (or I argue that the correct population of organisms not present) then simple deductions from lab-scale studies as quoted above cannot be extrapolated to commercial scale operations and certainly not by those kombucha producers who lack the knowledge to seriously dabble with such formulations, microbes and processes.

As acidity and ethanol levels are related we do recommend producers test alcohol frequently until they are sure as to how their systems work. Simpler assays are possible for routine monitoring and should be in place at most kombucha production facilities. That way only final samples for official or third party analysis can be tested or sent out for regulatory purposes using more sophisticated and accurate measuring instruments. [Alternatively, with a full understanding of the process and evaluation of final batch samples and alcohol content the data could be related back to acidity values – much more easily measured in-house - once consistent processes are in place.]

The final paper to mention discussing alcohol levels is by Kallel, *et al*. (25). Their paper deals with kinetics of sucrose disappearance and ethanol (measured enzymatically), acetate and cellulose production with green and black tea kombuchas. Stating 0.17 g alcohol yield per liter per day of fermentation which gives well less than 0.5% ABV using their prescribed laboratory-scale conditions. They do cite other work that shows 1.7 to 5.5 g/L ethanol (0.17-0.55% alcohol by weight) under similar experimental conditions. Even at such low levels they state: "These data could significantly impact the dietary habits of populations which forbid alcohol consumption" (25). Finally, I conjecture that the conversion of substrates to products on the whole may not give an immediately apparent true picture of the composition of products based on how they expressed the data. A much more careful look at ethanol production seems warranted here – especially in more up-scale production batches. However, this paper by Kallel, *et al*. (25) concludes with a warning about the effects of kombucha upon digestion in the human gut. While of interest in one dimension the portents are ominous in other directions. Building a clearer overview of the biochemistry of this fermentation process is needed. These authors (25) also discussed the drop in pH as fermentation takes place and noting that some manufacturers follow pH – allowing it to fall until it attains a pH value of 2.6-2.7 (see above for more on the significance of monitoring total acidity rather than pH here).

In conclusion, for section 1.5, in using simple model systems in highly controlled scientific situations, we note that the very few studies looking at ethanol levels might seem to be at odds with the generally held perception that kombucha fermentation leads to a more complete conversion of acids to ethanol. Our earlier review of the literature and calculations led this author to suspect that typical levels of ethanol would be between 0.75-1.25 percent by volume of cultures in routine practice. This is in fact a region over which many samples have been found when tested in our laboratory. Values above 2% are a little rarer but values close to 5% ABV have also been found among commercial ("non-alcoholic labeled") examples as obtained from local markets. The kombucha industry people want to believe they are creating products with less than 0.5% ABV. We beg to differ and the jury is still out regarding the ruling on this issue. Further studies using several officially accepted methods for alcohol determination need to be done on full-scale commercial batches of this complex beverage. Comparisons between the uses of green versus black teas have also received little attention though differences have been noted (25). Furthermore, the need to obtain the true sample specific gravity of samples in order to convert correctly any determined alcohol by weight values to true alcohol by volume values needs to be addressed. This issue of conversion seems to have been long forgotten by most experts in the industry and by those measuring alcohol levels in beverages. This will be significant in deciding if a beverage of the nature of complexity of kombucha is in fact at or below 0.5% ABV within the +/- 0.1% tolerances expected for the most accurate measuring instruments and methods available. [Note: in the review of the alcohol data discussed above only rough conversions from g/100 mL data to mL/100 mL for alcohol by volume values were possible – though likely quite valid approximations.]

1.6 Conclusions/Summary – Talking Point

There is rampant speculation in the industry that some components in Kombucha prevent the accurate determination of alcohol. This is in spite of the fact that several approved and official methods give similar results. Preliminary tests in our facility with spiking of known amounts of alcohol into Kombucha samples indicate no masking of this added alcohol when measured via densitometry and an NIR instrument. Further tests are needed but we believe that "masking" of alcohol (not seen by the instruments) nor enhancement of signals giving artefactually high readings is the case with the types and brands so far tested. If, however, any method is reading incorrectly (assuming it is done correctly in the first place with currently officially accepted methods and with required accuracy and precision) it is not affecting a decision by more than a fractional amount. This would not cause rejection of data in deciding if a kombucha is to be classed as alcoholic versus non-alcoholic based on the 0.5% ABV rule in effect in most countries. The tolerances at or close to 0.5% ABV might need to be re-addressed though not results indicating a far greater level of alcohol than say too far outside a range of 0.5-0.75% ABV. Our initial findings (and literature searches) indicated that many kombuchas would likely be between about 0.75 to 1.25% ABV. In fact we have found many to be in this general range. Moreover, these values are also congruent with some of the lab-scale studies discussed in section 1.5. Numerous examples on the current market are above this level. Nothing is preventing accurate readings in our opinion with official methods of analysis.

What the issue might be is a lack of understanding by kombucha producers of the nature and complexities of fermentation. And control of the culture (the composition of the tea-fungus or scoby) and fermentation, sanitary practices and aging conditions. Further research into the fermentation of such tea-fungus cultures is necessary, possibly followed by the establishment of new companies with microbiological production facilities in order to maintain scoby-type cultures. Tea-fungus specimens from different countries now having been shown to be radically different (discussed and referenced in the main text). This could help ensure healthy and correct maintenance of the complex commensal populations and strain identities of the organisms and help producers gain consistency and stability of production of their beverages. Cultures should not be supplied by shops and stores that do not understand microbiology or be passed from one home-brewer to another. Such cultures need to be defined with respect to the organisms present! This could help avoid contamination and potentially detrimental commensal population or "health" changes to this complex ecosystem and food-grade "ingredient". The Kombucha producer must not overlook the quality of the other raw materials used such as the tea, water, sugars or other fermentable ingredients. In the case of fruit flavored examples it should not be forgotten that whole fruits and damaged fruits especially are a potential source of a plethora of other microorganisms, including fermentative yeasts that might affect the global metabolic outcome of such beverages. They also provide a ready supply of other sugars and nutrients. In simple terms Kombucha production for commercial sale should be left to only a few individuals who are willing to learn the process and not simply because it looks like a lucrative opportunity. Fermentation and bottling needs to be done in specifically designated, environmentally controlled, facilities under food manufacturing best practices conditions and not in a kitchen or garage! Many dangers lurk for the unwary scoby user. The note in the article by O'Neill about comparing the scoby to the invasion of the body snatchers might in fact not be that far-fetched (1)!

References

1) O'Neill, M. (1994). A Magic Mushroom or Toxic Fad? New York Times. Dec 28, 1994; pg.C1.

2) Dufresne, C. and Farnworth, E. (2000). Tea, Kombucha, and health: a review. Food Research International. 33; 409-421.

3) Jayabalan, R., Malbasa, R. V., Loncar, E. S., Vitas, J. S. and Sathishkumar, M. (2014). A Review on Kombucha Tea – Microbiology, Composition, Fermentation, Beneficial Effects,

Toxicity, and Tea Fungus. Comprehensive Reviews in Food Science and Food Safety. 13; 538-550.

4) Marsh, A. J., O'Sullivan, O., Hill, C., Ross, R. P. and Cotter, P.D. (2014). Sequence-based analysis of the bacterial and fungal compositions of multiple kombucha (tea fungus) samples. Food Microbiology 38; 171-178.

5) Sievers, M., Lanini, C., Weber, A., Schuler-Schmid, U. and Teuber, M. (1995). Microbiology and Fermentation Balance in a Kombucha Beverage Obtained from a Tea Fungus Fermentation. System. Appl. Microbiol. 18; 590-594.

6) Reiss, J. (1994). Influence of different sugars on the metabolism of the tea fungus. Zeitschrift fur Lebensmittel-Untersuchung und-Forschung. 198; 258-261.

7) Malbasa, R., Loncar, E and Djuric, M. (2008). Comparison of the products of Kombucha fermentation on sucrose and molasses. Food Chemistry. 106; 1039-1045.

8) Malbasa, R., Loncar, E., Djuric, M. and Dosenovic (2008). Effect of sucrose concentration on the products of Kombucha fermentation on molasses. Food Chemistry. 108; 926-932.

9) Greenwalt, C.J. Steinkraus, K.H. and Ledford, R.A. (2000). Review: Kombucha, the Fermented Tea: Microbiology, Composition and Claimed Health Effects. Journal of Food Protection. 63(7); 976-981.

10) Jayabalan, R., Malini, K., Sathishkumar, M., Swaminathan, K. and Yun, S-E. (2010). Biochemical Characteristics of Tea Fungus Produced During Kombucha Fermentation. Food. Sci. Biotechnol. 19(3); 843-847.

11) Steels, H., James, S. A., Bond, C. J., Roberts, I. N and Stratford, M. (2002). *Zygosaccharomyces kombuchaensis*: the physiology of a new species related to the spoilage yeasts *Zygosaccharomyces lentus* and *Zygosaccharomyces bailii*. FEMS Yeast Research 2; 113-121.

12) Kurtzman, C. P., Robnett, C. J. and Basehoar-Powers, E. (2001). *Zygosaccharomyces kombuchaensis*, a new ascosporogenous yeast from "Kombucha tea". FEMS Yeast Research. 1; 133-138.

13) Battikh, H., Bakhrouf, A. and Ammar, E. (2012). Antimicrobial effect of Kombucha analogues. LWT – Food Science and Technology. 47; 71-77.

14) Liu, C.-H., Hsu, W.-H., Lee, F.-L, and Liao, C.-C. (1996). The isolation and identification of microbes from a fermented tea beverage, Haipao, and their interactions during Haipao fermentation. Food Microbiology, 13; 407-415.

15) Teoh, A.L., Heard, G. and Cox, J. (2004).Yeast ecology of Kombucha fermentation. Int. Journal of Food Microbiology. 95; 119-126.

16) Mo, H., Zhu, Y. and Chen, Z. (2008). Microbial fermented tea - a potential source of natural food preservatives. Trends in Food Science and Technology 19; 124-130.

17) Mayser, P., Fromme, S., Leitzmann, C. and Gründer, K. (1995). The yeast spectrum of the 'tea fungus Kombucha'. Mycoses. 38; 289-295.

18) Reva, O. N., Zaets, I. E., Ovcharenko, L. P., Kukharenko, O. E., Shpylova, S. P., Podolich, O. V., de Vera, J-P. and Kozyrovska, N.O. (2015). Metabarcoding of the kombucha microbial community grown in different microenvironments. AMB Express 5:35; (pages 1-8). [DOI 10.1186/s13568-015-0124-5]

19) Srinivasan, R. and Smolinske, S. (1997). Probable Gastrointestinal Toxicity of Kombucha Tea: Is This Beverage Healthy or Harmful? J. Gen. Intern. Med. 12; 643-644.

20) Blanc. P.J. (1996). Characterization of the Tea Fungus Metabolites. Biotechnology Letters. 18(2); 139-142.

21) Greenwalt, C.J., Ledford, R.A. and Steinkraus, K. H. (1998). Determination and Characterization of the Antimicrobial Activity of the Fermented Tea Kombucha. Lebensmittel-Wissenschaft und-Technologie. 31(3); 291-296.

22) Sreeramulu, G., Zhu, Y. and Knol. W. (2001). Characterization of Antimicrobial Activity in Kombucha Fermentation. Acta Biotechnol, 1; 49-56.

23) Jayabalan, R., Marimuthu, S. and Swaminathan, K. (2007). Changes in content of organic acids and tea polyphenols during kombucha tea fermentation. Food Chemistry. 102; 392-398.

24) Cvetkovic, D., Markov, S., Djuric, M., Savic, D. and Velicanski, A. (2008). Specific interfacial area as a key variable in scaling up Kombucha fermentation. Journal of Food Engineering. 85; 387-392.

25) Kallel, L., Desseaux, V., Hamdi, M., Stocker, P. and Ajandouz, E. H. (2012). Insights into the fermentation biochemistry of Kombucha teas and potential impacts of Kombucha drinking on starch digestion. Food Research International. 49; 226-232.

26) Fell, D. (1997). Understanding the Control of Metabolism. Portland Press.

Contact the Author: Gary Spedding. Brewing and Distilling Analytical Services, LLC.
www.alcbevtesting.com.
info@alcbevtesting.com.
859-278-2533

The BDAS, LLC laboratories are located in Lexington. KY. A triple TTB certified facility.