Understanding Kombucha fermentations: dynamic follow-up of a lab scale fermentation

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Introduction

At a time when consumers are more and more concerned about their diet and health and with a constantly increasing consumer demand for more natural and organic products, Kombucha is becoming a very popular drink in Western countries and as an alternative to sodas. Moreover, producers highlight the potential beneficial properties associated to the tea itself or the metabolites produced by the Kombucha microbiome. Kombucha is a naturally fermented beverage made from sweetened tea and is characteristically acidic and naturally fizzy due to metabolic activities of its complex microbial ecosystem (Figure 1). During fermentation, a biofilm is formed by acetic acid bacteria that floats on the surface of the tea and is very rich in yeast and acetic and lactic acid bacterial species. However, Kombucha, and more particularly biofilm formation and the creation of the complex microbial networks needed for fermentation, has not yet been well studied. Moreover, this biofilm is used in a backslopping process to start the next fermentation batch, which can impact overall product quality if changes in microbial populations occur.

In this study, a complex but controlled microbiota was used to dynamically follow lab scale fermentations. During the fermentation, several key parameters were followed including microbial populations (counts and metagenetics), physico-chemical (pH and density) and biochemical (organic acids, sugars, volatile compounds) parameters and, for the first time, biofilm formation by scanning electron microscopy and confocal microscopy.

Experimental strategy: Microbial cultures + Tea preparation → Fermentation in 400mL mini vessels → Incubation at 25°C, sampling at regular time intervals → Dynamic follow-up of the kombucha fermentations → Physico-chemical analyses / Microbiome / Biofilm structure / Metabolome

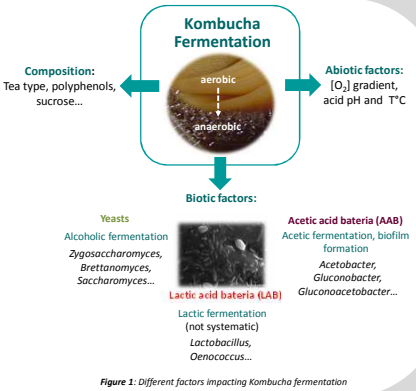


***Figure 1**: Different factors impacting Kombucha fermentation*

Microbial and physico-chemical dynamics

Microbial counts monitored over **27 days**:

in tea samples

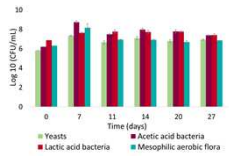


***Figure 2**: Microbial counts in tea samples during fermentation*

+2.5 log CFU/ml AAB counts
+1 log CFU/ml yeast counts
Population stable for LAB

in biofilm samples

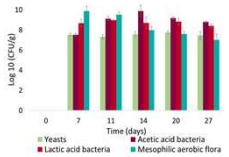


***Figure 3**: Microbial counts in biofilm samples during fermentation*

All 3 microbial groups well implanted in biofilm
+2 log CFU/g in AAB counts
AAB dominate in biofilms

Microbial activities associated with:

***Figure 4**: Thick biofilm formed by 27 days*

- biofilm **formation at a rate of** 0.34g/d for 7d then 0.15g/d up to 27d
- rapid decrease in **pH**
- rapid decrease in **density**
- **ethanol** production (<1%)
- metabolite **production**

Biofilm formation and microbial network

Biofilm observed by SEM and confocal microscopy at days 7, 11, 14, 21 & 27

Confocal microscopy observation of a biofilm at 11 days

***Sample preparation**: cells fixed with 1:1 PBS 1X:ice-cold ethanol 96% before hybridization with specific yeast and AAB/LAB FISH probes, in green and red, respectively, blue represents biofilm autofluorescence.*

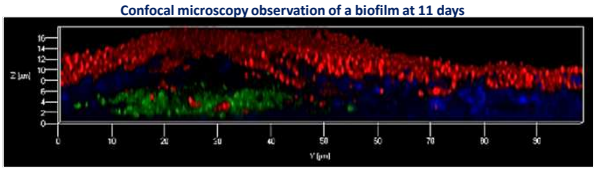


***Figure 5**: Confocal microscopy observation of a biofilm at 11 days fermentation*

14 day biofilm sample:

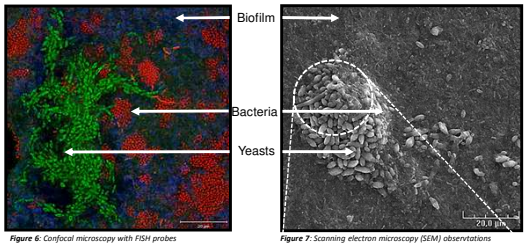
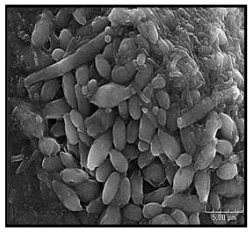


***Figure 6**: Confocal microscopy with FISH probes*

***Figure 7**: Scanning electron microscopy (SEM) observations*

***Sample preparation for SEM:** biofilm samples were treated with a cacodylate & 2.5% glutaraldehyde fixation solution then dehydrated using an ethanol bath before coating with a thin layer of metal.*



Complex network between bacteria and yeast:
- **yeast** in clusters
- **bacterial biofilm** network surrounds yeasts
- **biofilm** densifies over time

Biofilm potentially linked to their symbiotic relationship

Kombucha metabolome elucidated

Volatile metabolites determined by GC-MS (headspace)

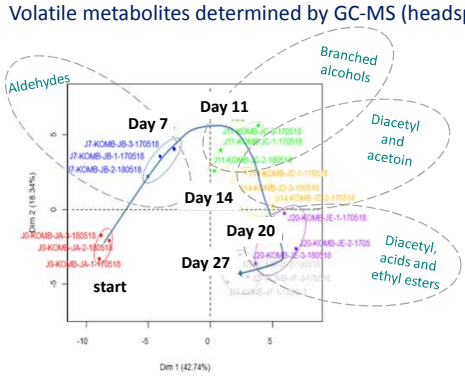
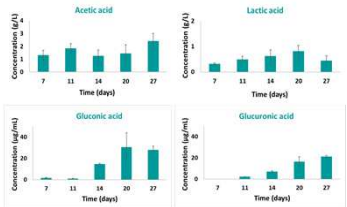


***Figure 8**: Principal component analysis on volatile metabolites during the lab scale kombucha fermentation*

Good repeatability of biological triplicates
Distinct changes in volatilome directly linked to fermentation time
Upcoming metagenetics data will help link species to functions

Organic acids determined by QTOF LC-MS and enzymatic kits



High concentrations of acetic acid linked to metabolically active AAB

Other organic acids vary in concentration and are mainly linked to AAB/LAB metabolism

Upcoming metagenetics should link these data to microbial species

Conclusions and perspectives

- **Acetic acid bacteria** actively participate to **biofilm** formation which surrounds other bacteria yeast over time
- Clear **symbiotic relationship between the different microbial groups** exists
- **Microbial species** involved in Kombucha fermentations **produce a specific metabolome over time**
- Upcoming metagenomics data should lead to better understanding of kombucha fermentation and product **quality**
- **Better understanding** the role of each microbial species leads to better management of microbial resources

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