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1 **Oxygen management during kombucha production: roles of the matrix, microbial**  
2 **activity, and process parameters.**

3 **Running Title: Oxygen management in kombucha**

4

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## 25 **Abstract**

26 Oxygen plays a key role in kombucha production, since the production of main organic acids, acetic  
27 and gluconic acids, is performed through acetic acid bacteria's oxidative metabolism. Oxygen  
28 consumption during traditional kombucha production was investigated by comparing kombucha to  
29 mono and cocultures in sugared tea of microorganisms isolated from kombucha. Two yeasts,  
30 *Brettanomyces bruxellensis* and *Hanseniaspora valbyensis* and one acetic acid bacterium  
31 *Acetobacter indonesiensis* were used. Results showed that tea compounds alone were mainly  
32 responsible for oxygen depletion during the first 24 hours following inoculation. During the first 7  
33 days phase of production in open vessel, the liquid surface was therefore the only access to oxygen for  
34 microorganisms, as anaerobic conditions were sustained below this area. During the 5 days second  
35 phase of production after bottling, comparison of cultures with different microbial compositions  
36 showed that oxygen was efficiently depleted in the head space of the bottles in 3 to 6 hours if the  
37 acetic acid bacterium was present. Lower access to oxygen after bottling stimulated ethanol production  
38 in *B. bruxellensis* and *H. valbyensis* cocultures with or without *A. indonesiensis*. This study provides  
39 insights into the management of oxygen and the roles of the tea and the biofilm during kombucha  
40 production.

## 41 **1. Introduction**

42 During the production of fermented beverages, the management of oxygen is an important parameter  
43 and can be either needed, monitored, or must be avoided. In wine production for example, contact with  
44 oxygen must be managed to avoid oxidation of polyphenols before and after alcoholic or malolactic  
45 fermentation, since soluble oxygen is being consumed by active metabolism (Ribéreau-Gayon *et al.*,  
46 2004). Microoxygenation can be used to bring a determined quantity of oxygen to improve yeast  
47 growth, but uncontrolled aeration can lead to the development of aerobic acetic acid bacteria, which  
48 are spoilage agent in the grape must matrix (Gomes *et al.*, 2018). Oppositely, aeration is a process  
49 used for vinegar production from wine, beer or cider by culturing acetic acid bacteria in aerated tanks  
50 (Lynch *et al.*, 2019). Traditional kombucha production can be seen as the incomplete acetification of a  
51 sugared tea infusion in static conditions (Villarreal-Soto *et al.*, 2018). A metabolic interplay between

52 symbiotic yeasts and acetic acid bacteria occurs in kombucha cultures. Starting from a sugared tea  
53 infusion, a kombucha culture is introduced under the form of liquid suspension or a cellulosic pellicle  
54 hosting live microorganisms as well (Tran *et al.*, 2020a). Kombucha cultures do not occur  
55 spontaneously but have to be obtained from a preexisting culture (Villarreal-Soto *et al.*, 2018). During  
56 the first phase of production in open vessel, acidification of the matrix is performed by acetic acid  
57 bacteria that oxidize ethanol and glucose into acetic acid and gluconic acid respectively using  
58 oxidative metabolism. Those substrates are made available by yeasts that break down sucrose into  
59 glucose and fructose through invertase activity and release ethanol through fermentation pathway.  
60 Parallely, a biofilm is formed at the surface of the liquid, mainly from acetic acid bacteria (Teoh *et al.*,  
61 2004; Tran *et al.*, 2021). A second phase occurs if natural carbonation is performed, by bottling the  
62 product in the presence of active microorganisms. At this stage, the limitation in oxygen is expected to  
63 inhibit oxidative metabolism of acetic acid bacteria and allows accumulation of ethanol and carbon  
64 dioxide since yeast alcoholic fermentation is still able to occur (Tran *et al.*, 2020b). So, as long as  
65 oxygen is accessible, ethanol content of kombucha remains low, but this is not the case after bottling if  
66 yeasts are still active. Since kombucha is typically sold as alcohol-free beverage, the control of the  
67 legal threshold of alcohol content (0.5 % (v/v) and 1.2 % (v/v) in the USA and European Union,  
68 respectively) is a major problem for the kombucha brewers (Office of the Federal Register, 1993;  
69 Official Journal of the European Union, 2011; Talebi *et al.*, 2017). The result of a controlled  
70 production is a beverage with a organoleptic profile comparable to a soda: sour, sweet and sparkling  
71 (Tran *et al.*, 2020a). In case of “overfermentation”, a tea vinegar is obtained (Chen and Liu, 2000).

72 So, oxygen appears to be a major process parameter to produce kombucha. Yet, it remains to be  
73 investigated, since, to the best of our knowledge, there are no studies on the consumption of oxygen  
74 during the production of kombucha. In our previous study, characterization of kombucha yeasts and  
75 acetic acid bacteria monocultures and cocultures in sugared tea medium was assessed and showed  
76 differences of behavior whether oxygen was available or not (Tran *et al.*, 2020b). The most striking  
77 effect could be observed for yeasts with different impacts on invertase and fermentative activities,  
78 depending on the species. For exemple, *B. bruxellensis* fermentative activity was efficient although

79 oxygen was accessible due to Custer effect (Custer, 1940). Oxygen limitation impacted mainly the  
80 production of gluconic acid by acetic acid bacteria, but **not** acetic acid production. It was hypothesized  
81 that the biofilm plays a role in the access to oxygen by allowing acetic acid bacteria to maintain  
82 themselves at the surface to perform oxidative metabolism in optimal conditions (May *et al.*, 2019;  
83 Schramm and Hestrin, 1954). This sessile state in aerobic conditions was also supposed to have an  
84 impact on the yeasts dynamics by favoring species over others or allowing better conditions for higher  
85 biodiversity compared to the planktonic state in the liquid (Coton *et al.*, 2017; Teoh *et al.*, 2004).  
86 However, another function of the biofilm could be that it acts as a lid, preventing dissolution of  
87 oxygen in the liquid phase, which would orientate yeasts metabolism towards fermentation (May *et al.*,  
88 2019).

89 The present study aims at investigating how and where oxygen is available during the production of  
90 kombucha. During the first phase, is it available in the liquid or only at the surface in the biofilm?  
91 How fast is it consumed during the second phase? How does oxygen availability impact microbial  
92 dynamics and metabolism? What is the role of the biofilm regarding the microorganisms' access to  
93 oxygen? To achieve it, experimental design was based on the comparison of monocultures and  
94 cocultures in sugared tea of microorganisms isolated from one control kombucha. Both production  
95 phases were investigated by analysis of microbial dynamics using culture dependent methods and  
96 chemical analyses. Oxygen content measurement was performed in the liquid and in the headspace  
97 using a non-intrusive method.

## 98 **2. Materials and Methods**

### 99 **2.1. Generation of kombucha, monocultures and cocultures of yeasts and acetic acid bacteria** 100 **isolated from kombucha.**

101 Sugared black tea, kombucha and cultures from microorganisms isolated from the same kombucha  
102 culture were produced according to a previous work (Tran *et al.*, 2020b) with some modifications. To  
103 produce sugared black tea, 1% (m/v) of tea was infused for one hour, then 50 g L<sup>-1</sup> of sucrose was  
104 added. Black tea (Pu'er Grade 1 TN4107) from Les Jardins de Gaïa (Wittisheim, France) was used.

105 The sugared black tea (SBT) obtained was used to produce kombucha as well as the cultures  
106 inoculated with isolated kombucha microorganisms. For the kombucha, 12% (v/v) of a 7-day black tea  
107 kombucha broth was added after SBT cooling. A primary inoculum was produced following the same  
108 procedure by using a mother culture obtained from the kombucha company Biomère (Paris, France) to  
109 ensure a physiological state of microorganisms comparable to regular industrial scale production. The  
110 mother culture is a kombucha culture refreshed monthly with sugared black tea.

111 Yeasts and acetic acid bacteria strains were isolated from the broth of the black tea kombucha  
112 mentioned in the last paragraph, as described in our previous study (Tran *et al.*, 2020b). Those  
113 microorganisms were selected according to their representation in terms of population during the  
114 elaboration of kombucha and their functionality (Tran *et al.*, 2020b). The selection includes:  
115 *Brettanomyces bruxellensis*, *Hanseniaspora valbyensis* and *Acetobacter indonesiensis*. The different  
116 modalities of monocultures and cocultures are detailed in Table 1.

117 Precultures of yeasts and the acetic acid bacterium were produced by restreaking material from stocks  
118 kept at -20°C on Wallerstein Lab (WL) agar (Hall, 1971) and De Man Rogosa and Sharpe (MRS) agar  
119 respectively, with an incubation at 28°C. Yeasts and acetic acid bacterium isolates were respectively  
120 inoculated in YPD liquid medium and MRS medium. Incubation occurred at 28°C in static conditions  
121 for 2 days in sterile vessels with untight caps to allow gas exchanges. After incubation, cells were  
122 washed with sugared black tea then centrifugated (3,000 g; 10 minutes at 4°C). Each population was  
123 inoculated at the rate of  $1.10^5$  cell mL<sup>-1</sup> in sugared black tea. The level of microorganisms was  
124 determined by flow cytometry using a BD Accuri C6 (Franklin Lakes, USA) with 0.1 µg mL<sup>-1</sup>  
125 propidium iodide to evaluate non-viable cells proportion (Stiefel *et al.*, 2015).

126 Cultures of 123 mL occurred in triplicates in 125 mL Boston flasks with a Specific Interfacial Surface  
127 (SIS) (Cvetković *et al.*, 2008) of 0.01 cm<sup>-1</sup> with bottlenecks loosely covered with tin fold to allow gas  
128 exchanges during 7 days (first phase P1) of production. Then, flasks were tightly capped for 5 more  
129 days of natural carbonation (second phase P2).

## 130 2.2. Microbiological analysis of liquid cultures and kombucha pellicles

131 Plate counting was performed to determine the populations of yeasts and/or acetic acid bacteria from  
132 the broth after inoculation (day 0), during P1 (days 1, 3, 5 and 7) and during P2 (days 10 and 12). Due  
133 to the absence of consistent cellulosic pellicle in cultures except for original kombucha (control),  
134 microbiology data regarding the kombucha pellicle only was included. To extract microorganisms  
135 from the pellicle, the whole 10 mm diameter biofilm was washed twice with 10 mL of sterile  
136 physiological water. Then, 10 mL sterile physiological water was added, and the pellicle was cut in  
137 bits using a sterile scalpel blade. Next, it was vortexed 3 minutes at full speed. This suspension was  
138 used as sample similarly to liquid culture samples. Successive decimal dilutions of samples were  
139 plated in triplicates on WL agar for yeasts and MRS agar for bacteria. Characterization of the yeasts  
140 on WL agar plates allowed the quantification of yeasts subpopulation according to the species based  
141 on morphological aspect, as described in our previous study (Tran *et al.*, 2020b). Identification of  
142 unknown or unclear yeast morphotype was performed as described in (Tran *et al.*, 2020b). After DNA  
143 extraction, 26S PCR was carried out using NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and  
144 NL4 (5'-GTCCGTGTTTCAAGACGG-3') primers (Maoura *et al.*, 2005). Amplified DNA was  
145 sequenced by Genewiz (Leipzig, Germany) according to Sanger method and identity was obtained by  
146 comparing the sequence to NCBI database. Results were expressed in CFU mL<sup>-1</sup> for liquid samples  
147 and in CFU g<sup>-1</sup> dry biofilm for biofilm extracts. Dry weight of biofilm was calculated at day 2, 3, 5 and  
148 7 using gravimetric method before and after drying at 102°C for 24 hours. The pellicles were  
149 discarded in the flasks just before P2.

### 150 **2.3. Chemical analyses**

151 Chemical analyses were performed at days 0, 1, 2, 3, 5, 7, 10 and 12. Cell-less samples were obtained  
152 by centrifugation (3,000g; 15 minutes, 4°C) and kept frozen at -20°C. Total acidity was determined by  
153 titration with 0.1 N NaOH and 0.2% phenolphthalein as color indicator. Acetic and succinic acids  
154 concentrations were determined at days 7 (end of P1) and 12 (end of P2) by HPLC as described  
155 previously (Tran *et al.*, 2020b). Sucrose, glucose and fructose, gluconic acid and ethanol  
156 concentrations were determined using enzymatic kits from Biosentec (Auzerville-Tolosane, France).

157 Results were expressed, according to the following formula (1):

$$158 \Delta Concentration = \Delta Endpoint concentration - \Delta Initial concentration \quad (1)$$

159 “Initial” corresponds to day 0 and the “endpoint” corresponds to the day of analysis.

160 Measurement of oxygen in the liquid and in the headspace of the culture flasks was performed using a  
161 Nomasense O2 P300 device and compatible Pst3 captors from Vinventions (Thimister-Clermont,  
162 Belgium). This device allows the quantification of headspace or soluble oxygen in liquids. Two  
163 captors per flask were placed on the inner part of the flask: one in the middle of the main body for  
164 measurement of soluble oxygen in the liquid and one in the bottleneck above the surface of the liquid  
165 to measure the oxygen in the headspace. Signals of captors could be read using the devices optical  
166 fiber from the outside of the flask, which make this analysis method non-intrusive. The device was  
167 calibrated according to user’s manual. Pst3 captors can accurately measure oxygen content up to 22  
168 mg L<sup>-1</sup> and has a lower detection limit of 15 µg L<sup>-1</sup>. Oxygen consumption was followed during the first  
169 eight hours after inoculation, then after 1, 2, 3, 5 and 7 days during P1. At P2, oxygen consumption in  
170 the headspace was followed during the first six hours following the capping of flasks, then at day 8, 10  
171 and 12. Oxygen partial pressure in the headspace was expressed in kPa. Those measurements were  
172 performed on separate samples than the ones described in part 2.1. and included initial saturation in  
173 oxygen using preliminary air injection for 10 minutes, but they were rigorously generated in the same  
174 way. A preliminary study determined the effect of sugar and tea in the medium without inoculation  
175 during the first eight hours following saturation.

## 176 **2.6. Statistical analyses**

177 All samples were made in triplicates. Confidence intervals were calculated using Microsoft Excel for  
178 Microsoft 365. Values were treated with ANOVA and Newman-Keuls pair test was applied in the case  
179 of significant difference ( $p < 0.05$ ) with R software (version 3.5.2.).

## 180 **3. Results and discussion**



### 181 3.1. Microbial dynamics

182 Plating allowed the quantification of yeast and bacteria population. Colony morphotyping using WL  
183 agar medium and complementary identification using 26S PCR allowed the discrimination of yeast  
184 populations according to genera and species. The microbial dynamics occurring in kombucha is  
185 described in Figure 1. In the liquid phase, total bacteria population increased from an average initial  
186 population of  $3.6 \cdot 10^4 \pm 1.8 \cdot 10^3$  CFU mL<sup>-1</sup> to a maximum population of  $9.2 \cdot 10^5 \pm 4.3 \cdot 10^5$  CFU mL<sup>-1</sup>  
187 reached on day 7 (Figure 1A). A similar behavior was observed for the yeast *B. bruxellensis* with an  
188 increase from initial population of  $5.2 \cdot 10^5 \pm 1.8 \cdot 10^4$  CFU mL<sup>-1</sup> to a maximum of  $1.1 \cdot 10^7 \pm 3.4 \cdot 10^5$  CFU  
189 mL<sup>-1</sup> on day 7. *H. valbyensis* population reached its maximum in a shorter time from initial population  
190 of  $3.5 \cdot 10^5 \pm 8 \cdot 10^3$  CFU mL<sup>-1</sup> to maximum of  $7.9 \cdot 10^6 \pm 1.2 \cdot 10^6$  CFU mL<sup>-1</sup> at day 3. No significant  
191 difference was detected before a significant decrease to  $3.2 \cdot 10^6 \pm 1.4 \cdot 10^6$  CFU mL<sup>-1</sup> at day 12. A  
192 similar behavior was observed in our previous study (Tran *et al.*, 2020b). It is noteworthy that in the  
193 liquid phase, yeasts population dominated the bacterial one with *B. bruxellensis* as dominant species.  
194 In contrast, bacteria dominated yeasts in terms of population in the biofilm from day 3. Starting from  
195 above  $8.6 \cdot 10^6$  CFU g<sup>-1</sup> dry biofilm at day 2 at the time of biofilm apparition, the population in yeasts  
196 and bacteria followed similar variations with a significant increase at day 3 ( $1.1 \cdot 10^9 \pm 2.4 \cdot 10^8$  and  $4.3$   
197  $\cdot 10^9 \pm 3.2 \cdot 10^8$  CFU g<sup>-1</sup> respectively), followed by significant decrease to  $2.1 \cdot 10^8 \pm 7.4 \cdot 10^7$  and  $4.3 \cdot 10^8 \pm$   
198  $3.2 \cdot 10^8$  CFU g<sup>-1</sup>, respectively (Figure 1B). No further variation was observed at day 7 before the  
199 biofilm was discarded with the end of P1. The growth of the biofilm in terms of dry weights showed a  
200 significant increase only between day 3 and day 5 from  $1.71 \pm 0.4$  mg to  $11.8 \pm 5.4$  mg. Consequently,  
201 the decrease in total microbial population per dry weight of biofilm between day 3 and day 5 can be  
202 explained by the production of exopolymers, mainly bacterial cellulose (Betlej *et al.*, 2020), and not  
203 by a microbial mortality. Yeasts dynamics in the biofilm involved the two species identified in the  
204 liquid phase (*B. bruxellensis* and *H. valbyensis*) and *Pichia occidentalis* (Figure 1C). This species was  
205 characterized by filamentous colonies and pear-shapes cells when not filamentous (pseudo-hyphae;  
206 data not shown). This yeast was the main species in terms of yeasts population in the biofilm, with a  
207 maximum absolute population at day 3 when total microbial population peaked. No other significant

208 variation in yeasts populations was detected in the biofilm. Results show that microbial dynamics  
209 between the liquid and the biofilm were distinct for kombucha, both between yeasts and bacteria and  
210 between yeast species in terms of dominance.

211 Figure 2 shows the population dynamics of monocultures and cocultures of microorganisms isolated  
212 from the kombucha culture mentioned before. Growth is shown per species in the different conditions  
213 and the effect of interaction are assessed by using the monoculture as a control. The kinetics of  
214 *B. bruxellensis* were similar in the monoculture and in the cocultures including *A. indonesiensis*  
215 (BBAI and T; Figure 2A). Maximum population was reached between day 4 and day 6 for the  
216 cocultures ( $5.3 \cdot 10^6 \pm 5.1 \cdot 10^5$  and  $4.2 \cdot 10^6 \pm 9.3 \cdot 10^5$  CFU mL<sup>-1</sup>, for BBAI and T respectively), and at  
217 day 7 for the monoculture ( $2.3 \cdot 10^6 \pm 4.5 \cdot 10^5$  CFU mL<sup>-1</sup>). However, the cocultures were equivalent or  
218 significantly higher in population at all points except at day 7 and day 12, which highlights a slightly  
219 better growth of *B. bruxellensis* when in presence of the acetic acid bacterium. In contrast,  
220 *B. bruxellensis* in yeasts coculture BBHV reached the stationary phase at day 1 and a maximum  
221 population at day 12 ( $2.8 \cdot 10^6 \pm 3.5 \cdot 10^5$  CFU mL<sup>-1</sup>). The presence of *H. valbyensis* without  
222 *A. indonesiensis* (BBHV) stimulated initial growth, but lowered population levels between day 3 and  
223 day 10 ( $1.3 \cdot 10^6 \pm 5.0 \cdot 10^5$  CFU mL<sup>-1</sup>). A different behavior could be observed for *H. valbyensis* (Figure  
224 2B). Cultures including *A. indonesiensis* (HVAI and T) showed identical growth between day 0 and  
225 day 3. Then, the presence of *A. indonesiensis* alone (HVAI) induced lower population levels, except at  
226 day 12 where maximum population was achieved for any condition ( $1.5 \cdot 10^7 \pm 5.4 \cdot 10^6$  CFU mL<sup>-1</sup>). On  
227 the contrary, the simultaneous presence of *A. indonesiensis* and *B. bruxellensis* (T) induced higher or  
228 equivalent population levels. This observation is also true for the yeasts coculture (BBHV), but with  
229 an additional boost of growth between day 0 and day 2, similarly to *B. bruxellensis*. In this culture,  
230 *H. valbyensis* dominated *B. bruxellensis* from day 2 to day 12. This dominance was also observable  
231 for T from day 0 to day 3, but after codominance was observed. Regarding *A. indonesiensis*, data  
232 showed that the presence of *H. valbyensis* stimulated the growth with the maximum population of  $6.4$   
233  $10^7 \pm 1.3 \cdot 10^7$  CFU mL<sup>-1</sup> (HVAI) at day 10, whereas the presence of *B. bruxellensis* (BBAI and T)  
234 induced equivalent or lower population levels (Figure 2C). In detail, *A. indonesiensis* population was

235 equivalent in monoculture and in BBAI from day 1 to day 3, then it showed similar behavior with T  
236 from day 7 to day 10. The population in T was always lower than in monoculture. To sum up, the  
237 presence of yeasts impacted the maximum population of *A. indonesiensis*, positively or negatively  
238 depending on the yeast species. However, *B. bruxellensis* had a dominant inhibiting influence and  
239 cancelled the effect induced by *H. valbyensis*. Yet, it is noticeable that T was the only culture in which  
240 no decrease in *A. indonesiensis* population was observed between day 10 and day 12. This decrease in  
241 population could be induced by the closing of the vessel during P2 and seemed to be prevented by the  
242 simultaneous presence of the two yeasts species. Difference in initial populations at day 0 might be  
243 due to known discrepancies between plate counting and flow cytometry, possibly because of viable  
244 non cultivable state (Millet and Lonvaud-Funel, 2000; Zou *et al.*, 2020). In all cases, the inoculation  
245 process was based on flow cytometry and rigorously the same for all conditions.

246 The comparison of cultures informed on the microbial dynamics of kombucha (Figures 1 and 2). The  
247 closest microbial composition with kombucha is the T culture that exhibited similar microbial  
248 dynamics with slight differences. The main common aspect is the yeasts dominance in the liquid phase  
249 and with the codominance of the two yeasts *B. bruxellensis* and *H. valbyensis* during most of P1. At  
250 P2, the decrease of *H. valbyensis* population was observed only in kombucha. Through the lens of the  
251 present results, the yeast dominance is due to *B. bruxellensis*. Additionally, it appears that the  
252 difference of initial growth between the two yeasts species (slower for *B. bruxellensis* and faster for  
253 *H. valbyensis*) was due to the presence of *A. indonesiensis* that cancelled the boost of growth provided  
254 by a yeast-yeast interaction, bringing initial dynamics back to those of yeast monocultures. The  
255 decrease in *H. valbyensis* and *A. indonesiensis* between day 10 and day 12 seemed to be induced by  
256 the closed vessel condition, but it might also involve other microbial interaction that could not be  
257 clearly determined experimentally. Namely, a possible influence of *P. occidentalis* from the biofilm to  
258 the liquid could be supposed. The study of this yeast species was not carried out due to its non-  
259 cultivability in our conditions of analysis.

### 260 **3.2. Oxygen consumption after inoculation (open vessel phase)**

261 The concentration in soluble oxygen was followed in the cultures and kombucha's liquid during the  
262 first phase of production P1 in open vessel. Focus was made on the first eight hours following the  
263 inoculation. One measurement per day was performed at day 1, 2, 3, 5 and 7 (Table 2). As preliminary  
264 study, the effect of sugar and tea on oxygen concentration in the liquid was assessed. Results show  
265 that no significant oxygen consumption occurred in water and sugared water, whereas important  
266 oxygen consumption occurred in sugared black tea infusion (SBT). After an initial fast decrease from  
267 saturation at 9.6 to 5.5 mg L<sup>-1</sup> in one hour, a second slower consumption occurred afterward from 4.9  
268 to 3.371 mg L<sup>-1</sup> after 2 and 8 hours, respectively. After 24 hours, the oxygen concentration was below  
269 0.1 mg L<sup>-1</sup>. This first result demonstrated that tea compounds alone induced a chemical consumption  
270 of oxygen in the liquid, leading to near anaerobic conditions after 24 hours. Spontaneous oxidation of  
271 tea polyphenols was likely the main phenomenon occurring (Cardoso *et al.*, 2020). Inoculated cultures  
272 except kombucha followed a similar behavior, but significant differences could be observed.  
273 Cocultures of *H. valbyensis* without *B. bruxellensis* (HV and HVAI) showed significantly higher  
274 oxygen concentrations than SBT, between one and five hours after inoculation. Oppositely, cocultures  
275 including both *B. bruxellensis* and *H. valbyensis* (BBHV and T) possessed lower oxygen  
276 concentrations than SBT. This highlights an effect of yeasts coculture with an increase in oxygen  
277 consumption regardless of the presence of *A. indonesiensis* (no significant differences between the two  
278 cultures). The behavior of kombucha was different from SBT and the other cultures with higher  
279 oxygen levels. This is probably due to the traditional method of inoculation involving kombucha from  
280 previous batch. The introduction of kombucha compounds might have changed oxygen solubility in  
281 the medium. Linear regression was applied to oxygen concentration curves between 2 and 8 hours  
282 after inoculation (Figure 3 and Table 2). Correlation coefficients (R<sup>2</sup>) for SBT was the weakest with  
283 0.87, whereas it was above 0.90 for all cultures. All inoculated cultures possessed greater slopes in  
284 absolute value than SBT except kombucha, with HV reaching the greatest value (-0.37). This slope  
285 was close to the one of BBHV (-0.35), which suggests the influence of *H. valbyensis*. The presence of  
286 *A. indonesiensis* implied greater slopes in absolute value for HVAI and T, compared to HV and BBHV  
287 respectively with values below -4.0. Microbial composition and activity appeared to affect oxygen

288 consumption kinetics during the first hours after inoculation, but it followed in any case a linear model  
289 after the initial fast drop occurring in the two first hours.

290 In general, highlighted differences had a small impact on the entire process, since after 24 hours, all  
291 cultures were near anaerobiosis except *B. bruxellensis* monoculture (BB). However, from 48 hours  
292 until the end of P1, all cultures including kombucha reached anaerobiosis with oxygen concentrations  
293 below 0.1 mg L<sup>-1</sup>. Consequently, presence of microorganisms had a very limited impact on oxygen  
294 concentration of sugared black tea in the first 24 hours following inoculation. Antioxidative  
295 compounds extracted from tea appeared to induce anaerobiosis in kombucha and suggests a matrix  
296 determinism, imposing oxygen access only at the surface of the liquid. It is noteworthy that no biofilm  
297 was present in any culture at that point. Anaerobiosis induction is therefore not attributable to the  
298 biofilm, but rather to the tea compounds of the matrix. Those are most probably tea polyphenols,  
299 which are major compounds in tea leaves highly sensitive to oxidation (Harbowy *et al.*, 1997).

### 300 **3.3. Oxygen consumption after bottling (closed vessel phase)**

301 All cultures achieved P1 in anaerobic conditions in the liquid phase, with oxygen access only at the  
302 surface of the liquid. Kombucha produced a consistent biofilm, whereas other cultures showed only  
303 inconsistent ones. After discarding the biofilm when present, all cultures were capped for the second  
304 phase that aims at natural carbonation in kombucha production (Tran *et al.*, 2020a). Oxygen  
305 consumption was followed in the headspace, during the first six hours following the capping of the  
306 flasks and during P2 (Table 3).

307 All cultures presented a decrease in headspace oxygen during the **first six** hours of P2. However,  
308 different behaviors could be observed in contrast to the beginning of P1. Regression was performed on  
309 the headspace oxygen concentration curves (Figure 4). A striking difference was visible among  
310 monocultures with linear regression associated to yeasts (BB and HV) and polynomial regression to  
311 *A. indonesiensis* (Figure 4A). Yeasts coculture BBHV was also associated to a linear regression with a  
312 greater slope in absolute value (-0.51). However, HV and BBHV possessed poor correlation  
313 coefficient (0.78 and 0.72 respectively). We can hypothesize that this linear oxygen consumption

314 consisted mainly in chemical oxidation as it was the case at the beginning of P1 (Table 2 and Figure 3).  
315 Nevertheless, the opposition yeast/acetic acid bacterium and linear/polynomial regression was also  
316 clearly illustrated in the yeast-acetic acid bacterium cultures and kombucha, which were all associated  
317 with polynomial regression. The fastest kinetics that induced anaerobiosis in the headspace (and thus,  
318 in the whole system) after three hours were kombucha and T. AI, BBAI and HVAI achieved this in six  
319 hours. This suggests a stimulating effect of the simultaneous presence of *B. bruxelensis* and  
320 *H. valbyensis* on *A. indonesiensis*' oxygen consumption kinetics. Consequently, the presence of acetic  
321 acid bacteria was the main driver of oxygen consumption during P2, and by extension during P1 most  
322 probably. Moreover, the decrease in *A. indonesiensis* population observed in AI, BBAI, HVAI (Figure  
323 2C) can be linked to the oxygen deprivation. It is remarkable that yeasts cultures (without acetic acid  
324 bacterium) did not achieve the same level of anaerobiosis during P2, with residual oxygen  
325 concentration above 1 kPa at day 12 (120 hours after capping). As a result, acetic acid bacteria were  
326 the main oxygen consumers during kombucha production and yeasts showed much lower consumption.  
327 Chemical analyses can bring elements about the microorganisms' metabolic activity in relationship  
328 with oxygen availability.

### 329 **3.4. Effect of oxygen concentration on basic chemical parameters of kombucha production**

330 Sucrose, glucose, and fructose variations suggested hydrolysis of sucrose and release of  
331 monosaccharides, yet no significant difference was detected between the days per sugar  
332 (Supplementary Material S1, S2 and S3).

333 Figure 5 shows the production kinetics of ethanol (Figure 5A) and total acidity (Figure 5B). Initial  
334 ethanol concentration at D0 was 0.15 g L<sup>-1</sup>, probably due to the use of pure ethanol for the disinfection  
335 of the sieve during sugared tea preparation. Surprisingly, the production of ethanol quickly reached  
336 +1.54 g L<sup>-1</sup> in only one day after inoculation for T, whereas other culture did not exceed +0.5 g L<sup>-1</sup>.  
337 *A. indonesiensis* in monoculture did not produce meaningful amounts of ethanol during the process, as  
338 expect, and likely consumed the initial ethanol of the medium through oxidative metabolism (Lynch *et*  
339 *al.*, 2019; Tran *et al.*, 2020b) (Figure 5A). On the other hand, yeasts in monocultures produced ethanol

340 during the process without exceeding  $1 \text{ g L}^{-1}$ . *B. bruxellensis* showed greater fermentative activity than  
341 *H. valbyensis*, which was reported in our previous study (Tran *et al.*, 2020b). This was especially true  
342 during P2 which suggests a stimulation of fermentation from oxygen exhaustion (Figure 4). This  
343 phenomenon was especially visible for the yeasts coculture BBHV with a pronounced increase of  
344 ethanol production between day 7 and day 10 (from  $+0.11$  to  $+3.36 \text{ g L}^{-1}$ ). Increase in ethanol  
345 production also occurred in T with a lower intensity ( $+0.57 \text{ g L}^{-1}$ ). This difference depending on the  
346 presence of acetic acid bacteria can be explained by their capacity to oxidize ethanol even with limited  
347 oxygen (Chouaia *et al.*, 2014; Tran *et al.*, 2020b). It is visible on the acidification kinetics (Figure 5B).  
348 Total acidity increased in all cultures, but different behavior could be observed. Yeasts in monoculture  
349 (BB and HV) and the yeasts coculture BBHV did not show significant increase in total acidity  
350 production between day 3 and day 7. Oppositely, it was the case for cultures including  
351 *A. indonesiensis* (AI, BBAI, HVAI, T) and kombucha. As expected, acidification occurred during P1.  
352 However, as suggested by the ethanol production kinetics, acidification also took place during P2.  
353 Three groups could clearly be statistically distinguished: a low acidification group with HV and HVAI,  
354 an intermediate acidification group with AI, BB and BBHV and a higher acidification group with  
355 BBAI, T and kombucha. T reached significantly higher total acidity increase with an average value of  
356  $30.7 \text{ meq L}^{-1}$ .

357 To get more insight into the acidification process, individual organic acids were analyzed in the  
358 samples (Table 4). Acetic and succinic acids were detected in all cultures except BB at day 7 (but  
359 detected at day 12), whereas gluconic acid was only detected in cultures including acetic acid bacteria  
360 (AI, BBAI, HVAI, T and kombucha), as expected (Lynch *et al.*, 2019). Cultures with low acidification  
361 capacity (HV and HVAI) showed no significant increase in any of the organic acids. Indeed, in our  
362 previous study, *H. valbyensis* was characterized with low invertase and fermentative activity, which  
363 prevented the release of substrate for acetic acid bacteria and thus an efficient acidification (Tran *et al.*,  
364 2020b). The intermediate group cultures (BB, AI and BBHV) performed significant acidification  
365 between day 7 and day 12 through significant production of acetic acid (from no detection to  $+0.36 \text{ g}$   
366  $\text{L}^{-1}$  for BB, from  $+0.46$  to  $+0.82$  for AI and from  $+0.14$  to  $+0.51 \text{ g L}^{-1}$  for BBHV). As it is expected

367 from *A. indonesiensis* to produce acetic acid, *B. bruxellensis* is also known to produce acetic acid,  
368 particularly in aerated conditions due to Custer effect (Aguilar Uscanga *et al.*, 2003; Custer, 1940;  
369 Steensels *et al.*, 2015). *A. indonesiensis* in monoculture also produced more gluconic acid, which was  
370 also reported previously (Tran *et al.*, 2020b). Finally, the group with high acidification capacity  
371 included BBAI, T and more specifically, kombucha, with a significant increase in acetic acid between  
372 day 7 and day 12 (from +1.27 to +1.80 g L<sup>-1</sup>). This might imply a production of other organic acids  
373 during P2 for BBAI and T. Acidification during P1 in kombucha was mainly due to acetic acid  
374 production from acetic acid bacteria. However, acidification from acetic acid bacteria oxidative  
375 metabolism carried on regardless of limited oxygen of the headspace, which was consumed in few  
376 hours. As stated in our previous study, production of acetic acid could occur from pathways that are  
377 not dependent on oxygen (Prust *et al.*, 2005; Tran *et al.*, 2020b). Moreover, oxygen limitation did not  
378 only stimulate fermentation but also production of organic acids that participated in the acidification  
379 during P2.

380 To summarize, after inoculation of kombucha in sugared tea, antioxidant tea compounds (presumably  
381 polyphenols, including flavan-3-ols (Harbowy *et al.*, 1997; Tran *et al.*, 2022)) spontaneously  
382 consumed oxygen within 24 hours (Table 2, Figure 3). At this point, oxygen consumption was mainly  
383 chemical since microbial population and activity were very limited in the first hours following  
384 inoculation. Once anaerobiosis was achieved, the liquid phase remained an anaerobic medium for the  
385 rest of the production process. This is expected to orientate yeasts toward fermentative metabolism  
386 and thus the release of ethanol (Figure 5). Together with monosaccharides, those compounds were  
387 converted into organic acids by acetic acid bacteria through oxidative metabolism (Table 4). This  
388 metabolism can be optimally performed only at the surface of the liquid, independently of the biofilm  
389 formation (Figure 1 and 2). The biofilm's function would be as a catalyser by keeping yeasts and  
390 bacteria at the same location (Tran *et al.*, 2021), but it is not the driver of anaerobiosis in the liquid, as  
391 previously suggested (May *et al.*, 2019). So, this might explain the bacteria domination in the aerobic  
392 biofilm and the yeast domination in the anaerobic liquid. Our results suggested that bacteria's  
393 oxidative metabolism was the main factor maintaining anaerobiosis in the liquid during P1 and the



394 rapid consumption (few hours) of the headspace oxygen at the beginning of P2 (Figure 4), leading to  
395 complete anaerobiosis of the system. This stimulated ethanol production by yeasts and lead to ethanol  
396 accumulation due to inhibition of oxidative metabolism of acetic acid bacteria. However, this  
397 inhibition was partial and acidification from acetic acid bacteria remained active. The genomic study  
398 of acetic acid bacteria suggests that the capacity to thrive at low oxygen concentration is necessary for  
399 them to act as a symbiote in the insects' gut (Chouaia *et al.*, 2014). Our data also showed that P2  
400 stimulated the production of organic acids by yeasts (namely acetic acid) from *B. bruxellensis*, that  
401 could contribute to the acidification during P2. At the end of P2, the decrease in population of  
402 *A. indonesiensis* and *H. valbyensis* could point at a delayed negative effect of P2 on their populations  
403 (Figure 1).

## 404 **5. Conclusions**

405 This study provides a better understanding of the fate of oxygen in the context of kombucha  
406 production. It brings useful information on the oxygen consumption of kombucha yeasts and acetic  
407 acid bacteria and their kinetics of consumption. These data can be linked to both microbial dynamics  
408 and activities and can help kombucha brewers to manage oxygen input during fermentation. In the  
409 context of traditional static production, this study highlights the importance of Specific Interfacial  
410 Surface (SIS), meaning the ratio between the liquid surface and the liquid volume. The SIS conditions  
411 the access to oxygen since the liquid is an anaerobic medium except at the surface during the first  
412 phase of production. Tea polyphenols appear to consume the oxygen in the liquid within 24 hours  
413 regardless of the addition of microorganisms. Therefore, this study also confirms that the biofilm is  
414 not the origin of the liquid's anaerobiosis. The biofilm's location at the liquid/air interface is imposed  
415 by the matrix and microbial activity's oxygen consumption since it is the zone where oxidative  
416 metabolism is the most active. By extension, the matrix also sets conditions to orientate microbial  
417 dynamics and activities, depending on the utilization of oxygen. Finally, more insights could be  
418 obtained about the restriction in oxygen access induced by the second phase of production, which  
419 remains under-investigated and pose issues of reproducibility during production. Complementary to  
420 oxygen, the management of carbon dioxide and gas pressure would be very useful to enhance

421 knowledge on natural carbonation of kombucha. Also, it could be interesting to determine to what  
422 extent maintaining an anaerobic environment could protect beneficial compounds of kombucha that  
423 are sensitive to oxidation, typically tea polyphenols.

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#### 427 **Declaration of interest**

428 Authors declare no conflict of interest.

#### 429 **Author contributions**

430 Thierry Tran took the lead of the writing of this article, but all other authors provided critical and  
431 complementary elements to the manuscript. Thierry Tran performed microbiological and chemical  
432 analyses, as well statistical treatments. François Verdier and Antoine Martin provided the kombucha  
433 cultures used in the experiments.

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527

529 Table 1: Description of the different cultures.

	Monocultures in sugared black tea	Yeast-Yeast coculture in sugared black tea	Yeast(s)-acetic acid bacteria cocultures (minimal consortia) in sugared black tea	Black tea kombucha (detected microorganisms in the liquid)
Microbial composition (Code)	<i>B. bruxellensis</i> (BB) <i>H. valbyensis</i> (HV) <i>A. indonesiensis</i> (AI)	<i>B. bruxellensis</i> and <i>H. valbyensis</i> (BBHV)	<i>B. bruxellensis</i> and <i>A. indonesiensis</i> (BBAI)  <i>H. valbyensis</i> and <i>A. indonesiensis</i> (HVAI)  <i>B. bruxellensis</i> , <i>H. valbyensis</i> and <i>A. indonesiensis</i> (T)	<i>B. bruxellensis</i> , <i>H. valbyensis</i> , <i>Saccharomyces cerevisiae</i> , and bacteria including <i>A. indonesiensis</i> , <i>Acetobacter papayae</i> and <i>Komagataeibacter</i> <i>saccharivorans</i> (Kombucha)

530

531 Table 2: Oxygen concentration of the liquid in non-inoculated and inoculated media for 24 hours  
532 (average values, n = 3) and linear regression parameters between 2 and 8 hours. Common letters  
533 correspond to no significant differences between values of the same time of analysis according to  
534 ANOVA ( $\alpha = 0.05$ ). Bold values highlight significant difference with sugared black tea control  
535 (SBT). BB = *B. bruxellensis*, HV = *H. valbyensis*, AI = *A. indonesiensis*. T corresponds to the  
536 coculture of all three microorganisms. nd = not determined.

in mg L <sup>-1</sup>	Time (hours)										Linear regression parameters (y = ax + b)			
	0	1	2	3	4	5	6	7	8	24	48 to 168	a	B	R <sup>2</sup>
Water	9.2a	8.5a	8.5a	8.5a	8.4a	8.4a	8.4a	8.4a	8.4a	8.4a	nd	nd	nd	nd
Sugared Water	9.3a	8.5a	8.5a	8.4a	8.4a	8.3a	8.3a	8.3a	8.3a	nd	nd	nd	nd	nd
SBT	9.6a	5.5c	4.9d	4.4d	3.8d	3.6d	3.5cd	3.4c	3.3c	0.1b	nd	0.25	5.09	0.87
BB	9.6a	5.4c	4.6d	<b>4.1e</b>	<b>3.6de</b>	3.3de	3.0de	2.9cd	2.7cd	<b>1.6a</b>		0.31	5.01	0.95
HV	9.6a	<b>6.5b</b>	<b>5.7c</b>	<b>4.9c</b>	<b>4.4c</b>	<b>4.0c</b>	3.7c	3.5c	3.5c	0.1b		0.37	6.08	0.92
AI	9.6a	5.3c	4.6d	<b>4.1e</b>	3.6de	3.4de	3.1de	2.9cd	2.8cd	0.2b		0.30	4.98	0.95
BBAI	9.6a	5.3c	4.6d	<b>4.1e</b>	3.7de	3.4de	3.1de	2.9cd	2.8cd	0.9ab	< 0.1	0.30	4.98	0.95
HVAI	9.6a	<b>6.5b</b>	<b>5.6c</b>	<b>4.9c</b>	<b>4.4c</b>	<b>3.9c</b>	3.6cd	3.3c	3.1cd	0.1b		0.42	6.19	0.96
BBHV	9.6a	5.4c	4.5d	<b>3.8e</b>	<b>3.5ef</b>	<b>3.0ef</b>	<b>2.8ef</b>	<b>2.5de</b>	<b>2.4de</b>	0.1b		0.35	4.98	0.95
T	9.6a	5.1c	4.4d	<b>3.9e</b>	<b>3.5f</b>	<b>2.9f</b>	<b>2.5f</b>	<b>2.1e</b>	<b>1.8e</b>	0.1b		0.44	5.20	0.99
Kombucha	9.6a	<b>6.2b</b>	<b>6.1b</b>	<b>6.0e</b>	<b>6.0b</b>	<b>5.7b</b>	<b>5.6b</b>	<b>5.3b</b>	<b>5.2b</b>	0.1b		0.17	6.53	0.95

537 **Table 3: Oxygen concentration of the headspace of cultures during the next 48 hours and after**  
 538 **120 hours after bottling (average values, n = 3). Common letters correspond to no significant**  
 539 **differences between values of the same time of analysis according to ANOVA ( $\alpha = 0.05$ ). BB =**  
 540 ***B. bruxellensis*, HV = *H. valbyensis*, AI = *A. indonesiensis*. T corresponds to the coculture of all**  
 541 **three microorganisms.**

Time after capping (hours)	Oxygen Partial Pressure (kPa)							
	0	0.5	1	3	6	24	72	120
<b>BB</b>	21.4ab	21.5a	21.8a	20.5a	19.6a	14.3a	7.5a	1.5a
<b>HV</b>	21.2ab	21.8a	21.7a	20.7a	20.3a	13.1a	5.3a	1.1a
<b>AI</b>	21.6a	17.9b	15.1b	5.8b	0.5b	0.1b	0.1a	0.1a
<b>BBAI</b>	18.4c	14.9b	8.4cd	4.9b	1.3b	0.1b	0.8a	0.3a
<b>HVAI</b>	17.9c	15.5b	10.7c	6.2b	0.7b	0.1b	0.3a	0.3a
<b>BBHV</b>	20.5	22.1a	20.2a	19.0a	18.2a	14.0a	7.9a	1.4a
<b>T</b>	17.7c	10.5c	6.0d	1.4b	1.0b	0.1b	0.1a	0.1a
<b>Kombucha</b>	20.5ab	18.1b	10.3c	0.9b	0.1b	0.1b	0.2a	0.1a

542

543 **Table 4: Difference in organic acids in cultures between day 0, day 7 and day 12 (average values,**  
 544 **n = 3). *B. bruxellensis* (BB), (**B**) *H. valbyensis* (HV) and *A. indonesiensis* (AI). T corresponds to**  
 545 **the coculture of all three microorganisms. nd = not detected. Common letters correspond to no**  
 546 **significant differences between values of the same time of analysis according to ANOVA ( $\alpha =$**   
 547 **0.05). Bold police highlight significant difference between day 7 and day 12 for a given culture.**

	Acetic acid (g L <sup>-1</sup> )		Gluconic acid (g L <sup>-1</sup> )		Succinic acid (g L <sup>-1</sup> )	
	Day 7	Day12	Day 7	Day12	Day 7	Day12
<b>BB</b>	nd	<b>0.36cdef</b>	nd	nd	0.25a	0.29a
<b>HV</b>	0.05fg	0.17defg	nd	nd	0.02a	0.88a
<b>AI</b>	<b>0.46cde</b>	<b>0.82b</b>	<b>0.26b</b>	<b>0.35a</b>	0.10a	0.06a
<b>BBAI</b>	0.24cdefg	0.39cdef	0.10c	0.05c	0.17a	0.22a
<b>HVAI</b>	0.22cdefg	0.35cdef	0.07c	0.03c	0.07a	0.08a
<b>BBHV</b>	<b>0.14efg</b>	<b>0.51cd</b>	nd	nd	0.54a	1.05a
<b>T</b>	0.29cdefg	0.38cdef	0.08c	0.07c	0.34a	0.47a
<b>Kombucha</b>	<b>1.27c</b>	<b>1.80a</b>	0.21b	0.23b	0.93a	1.10a

548

549

550 **Figures Caption**

551 **Figure 1: Microbial dynamics of kombucha production (A) in the broth, (B) in the biofilm with**  
552 **(C) focus on the yeast species (average values, n = 3). Curves are meant to ease the reading. The**  
553 **dark background corresponds to closed vessel phase. Error bars correspond to confidence**  
554 **intervals ( $\alpha = 0.05$ ). Common letters correspond to no significant differences between values**  
555 **according to ANOVA ( $\alpha = 0.05$ ).**

556 **Figure 2: Comparison of microbial dynamics per microorganism during open and closed vessel**  
557 **phases (average values, n = 3) for (A) *B. bruxellensis*, (B) *H. valbyensis* and (C) *A. indonesiensis***  
558 **(AI) in the different cultures: BB, HV, AI, BBAI, HVAI, BBHV and T. BB = *B. bruxellensis*, HV**  
559 **= *H. valbyensis*, AI = *A. indonesiensis* and T corresponds to the coculture of all three**  
560 **microorganisms. Curves are meant to ease the reading. The dark background corresponds to**  
561 **the closed vessel phase. Error bars correspond to confidence intervals ( $\alpha = 0.05$ ).**

562 **Figure 3: Soluble oxygen consumption in the liquid for (A) non inoculated media, (B)**  
563 **monocultures and yeast coculture and (C) minimal consortia and kombucha cultures. Pointed**  
564 **lines show linear regression of the oxygen consumption kinetics with corresponding equation ( $y$**   
565 **=  $ax + b$ ) and correlation coefficient ( $R^2$ ). SBT = sugared black tea, SW= sugared water, W =**  
566 **water, BB = *B. bruxellensis*, HV = *H. valbyensis*, AI = *A. indonesiensis*. T corresponds to the**  
567 **coculture of all three microorganisms.**

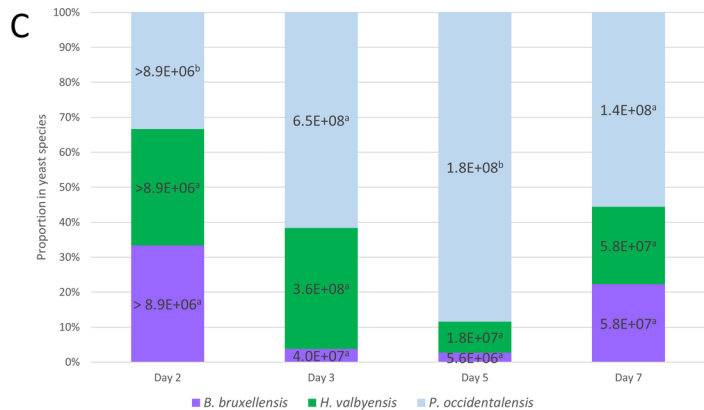
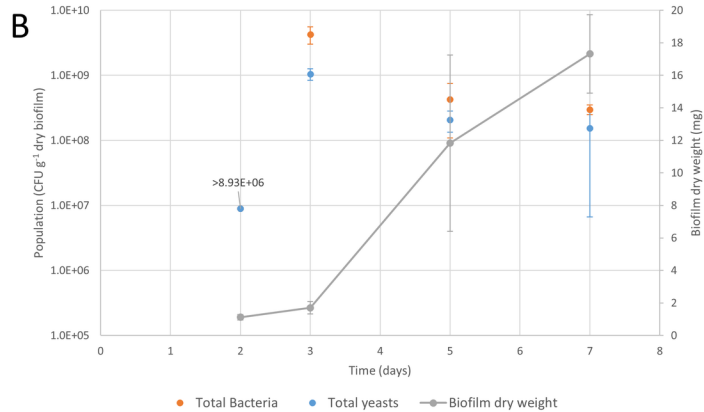
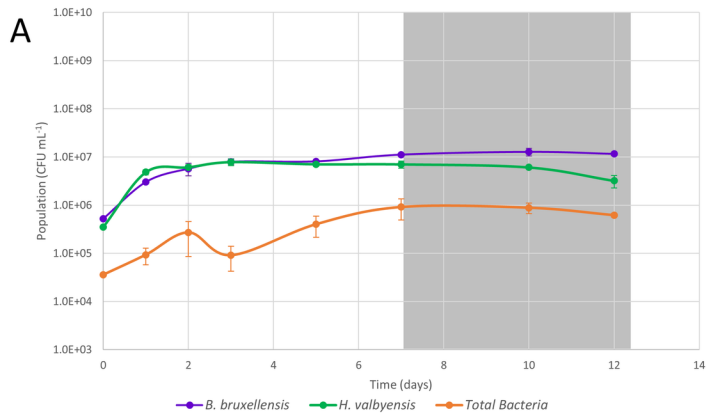
568 **Figure 4: Oxygen consumption in the headspace for (A) monocultures and yeast coculture and**  
569 **(B) minimal consortia and kombucha cultures. Pointed lines and curves show regression of the**  
570 **oxygen consumption kinetics with corresponding equation ( $y = ax + b$  for linear regression and  $y$**   
571 **=  $ax^2 + bx + c$  for polynomial regression) and correlation coefficient ( $R^2$ ). BB = *B. bruxellensis*,**  
572 **HV = *H. valbyensis*, AI = *A. indonesiensis*. T corresponds to the coculture of all three**  
573 **microorganisms.**

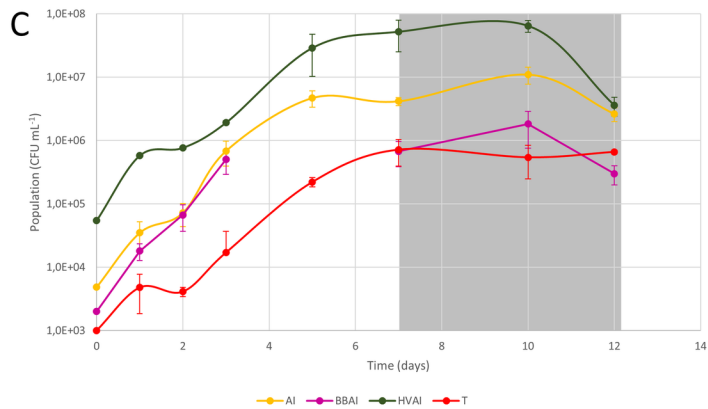
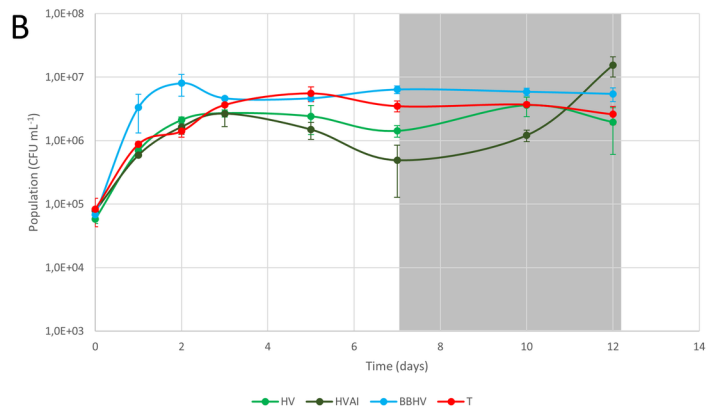
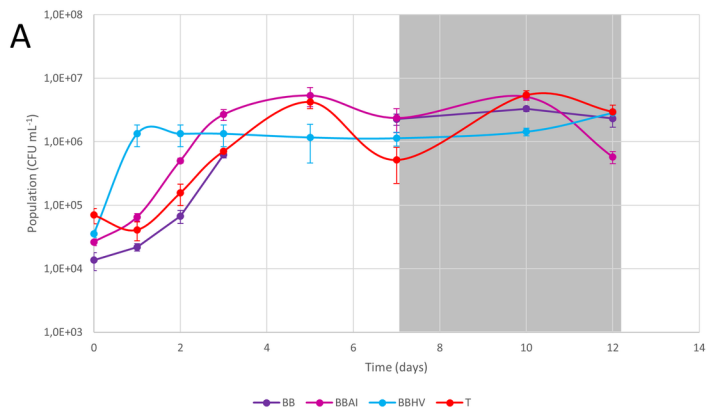
574 **Figure 5: Difference in (A) ethanol and (B) total acidity in cultures between day 0 and the**  
575 **endpoint (day 1, 3, 5, 7, 10 or 12) during the two-phase kombucha production. *B. bruxellensis***

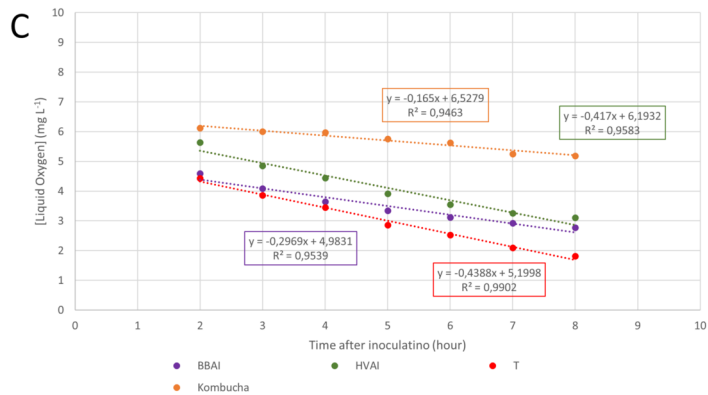
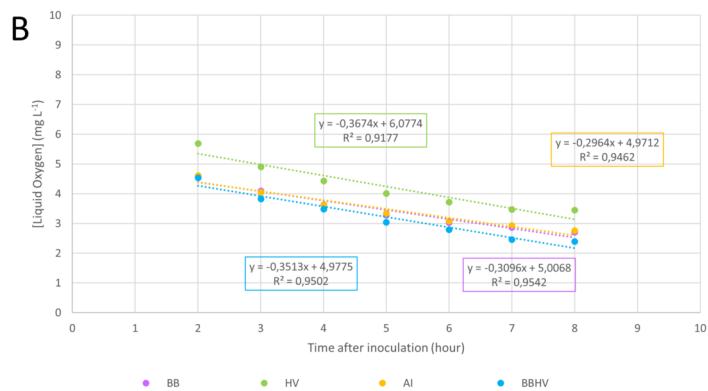
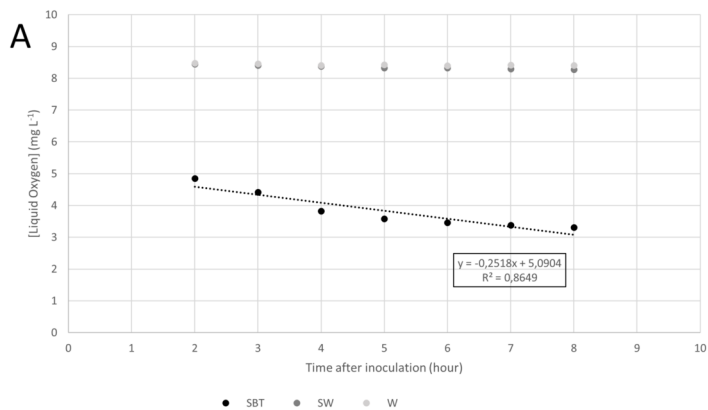
576 **(BB), (B) *H. valbyensis* (HV) and *A. indonesiensis* (AI). T corresponds to the coculture of all**  
577 **three microorganisms. Curves are meant to ease the reading. The dark background corresponds**  
578 **to the closed vessel phase. Error bars correspond to confidence intervals ( $\alpha = 0.05$ ).**

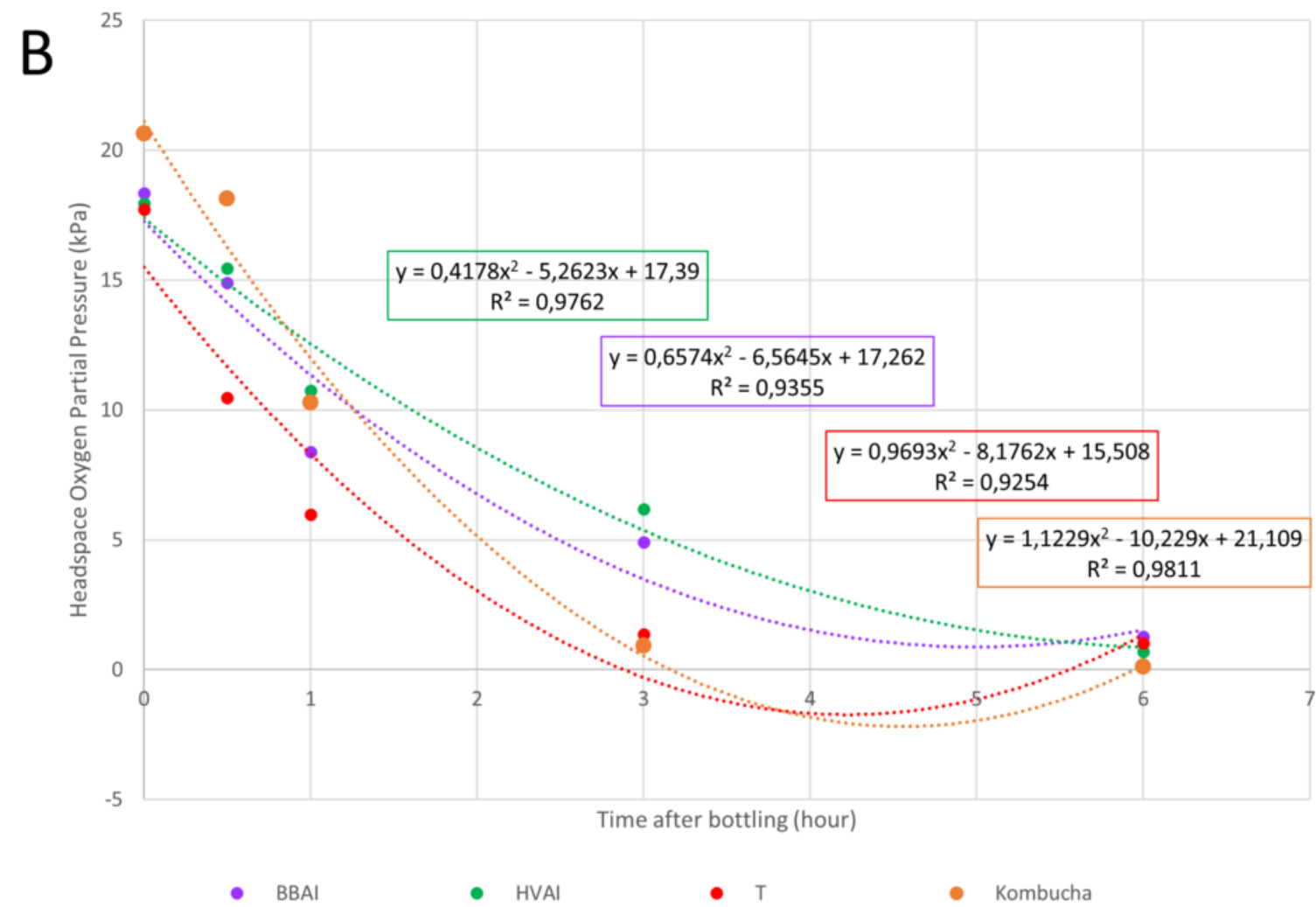
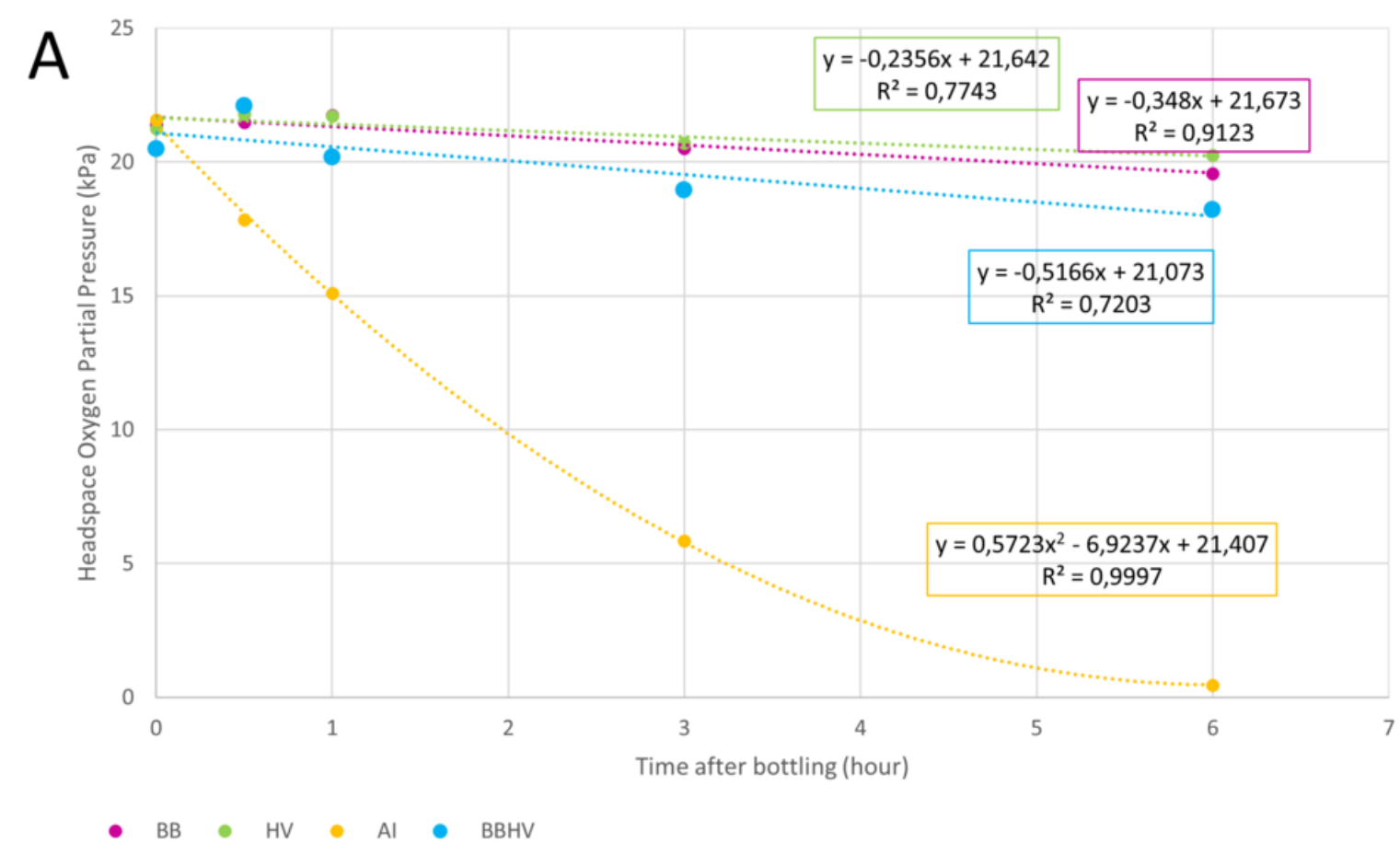
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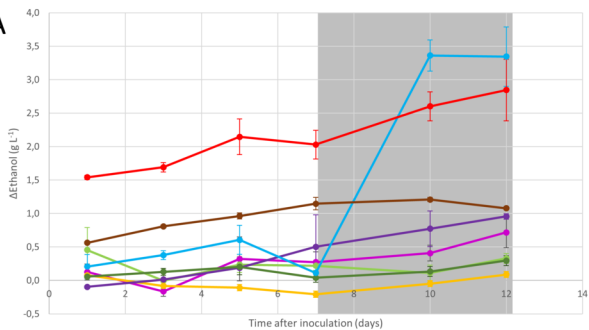






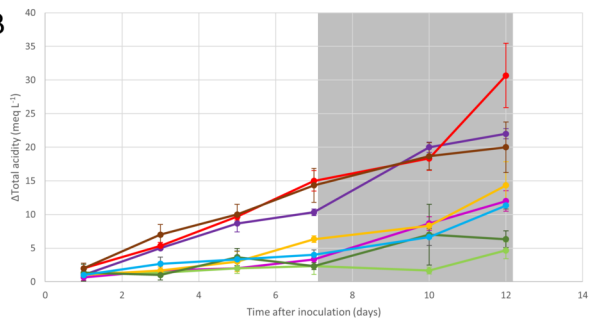


A



BB HV AI BBAI HVAI BBHV T Kombucha

B



BB HV AI BBAI HVAI BBHV T Kombucha

